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Globulin Complexes with Oestrogenic Acids

By W. HAUSMANN (Gnehm Stiftung Scholar)
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(*Received* 3 *May* 1948)

Harington and his colleagues (Clutton, Harington & Yuill, 1938; Butler, Harington & Yuill, 1940) started a series of studies on the immunological properties of certain protein complexes containing simple physiologically active substances as haptene groups. They showed that immunization with a thyroxine-protein complex produced resistance to the action of thyroxine. Similarly, immunization with an aspirin-protein complex produced resistance to the action of aspirin.

We have prepared the globulin complexes of (*a*) the 7-methyl ether of racemic bisdehydrodoisynolic acid (7-methoxy-2-methyl-1-ethyl-1:2:3:4-tetrahydrophenanthrene-2-carboxylic acid) and of (*b*) the 6-methyl ether of αα-dimethyl-β-ethyl-allenolic acid (2-(6′-methoxy-2′-naphthyl)-1:1-dimethyl-*n*-valeric acid, Horeau & Jacques, 1947) with a view to determining whether immunization with the bisdehydrodoisynolic and the allenolic acid protein complexes will produce resistance to the bisdehydrodoisynolic and the allenolic acids themselves, and also to other uncombined oestrogens. Expressed schematically, it was desired to discover whether immunization with a globulin complex of oestrogenic acid *A* will confer resistance to the action of acid *A* as well as to oestrogenic acid *B* and other oestrogens. It was hoped that such a series of experiments would throw light on the part played by physiological properties and chemical constitution in the conferment of immunological properties on protein complexes.

The method we have used for coupling the oestrogenic acids with globulin has been that used by Butler *et al.* (1940) for coupling acetylsalicylic acid with globulin. The chlorides of both acids were treated at 0° in aqueous acetone solution with sodium azide to give the *azides*, which in both cases were very unstable to heat. These were then treated with horse-serum globulin under alkaline conditions.

EXPERIMENTAL

7-*Methyl ether of bisdehydrodoisynolic acid-globulin complex*

Chloride of 7-methyl ether of bisdehydrodoisynolic acid. The racemic 7-methyl ether of bisdehydrodoisynolic acid, m.p. 229–230°, was kindly supplied by Dr K. Miescher. The acid chloride was prepared according to Anner, Heer & Miescher (1946) using oxalyl chloride (Staudinger, 1908). After two crystallizations from benzene-light petroleum (b.p. 60–80°), colourless prisms (yield 80% of theory) were obtained, melting at 148–149°. (Found: C, 71·9; H, 6·6; Cl, 11·1. Calc. for $C_{19}H_{21}O_2Cl$: C, 72·0; H, 6·7; Cl, 11·2%.)

Azide. To a solution of 320 mg. of acid chloride in 18 ml. of acetone 80 mg. (1·25 mol.) of NaN_3 in 2·5 ml. of water were added and the clear reaction mixture was kept at 0° for 3 hr. The solution was then diluted with 50 ml. of ice water, when a very fine white precipitate formed. On extraction with cold ether, drying with $CaCl_2$ at 0° and evaporation to dryness in N_2 under reduced pressure without application of heat, 300 mg. (94% of theory) of colourless *azide* were obtained, melting sharply at 88° with evolution of N_2. The azide is fairly stable at 0°, but loses N_2 on recrystallization from low-boiling solvents. Therefore, the freshly prepared crude product was dried in high vacuum for 1 hr. and analyzed. (Found: C, 70·4; H, 6·6; N, 13·4. $C_{19}H_{21}O_2N_3$ requires: C, 70·5, H, 6·5; N, 13·0%.)

Horse-serum globulin solution. This was prepared by treating 600 ml. of fresh horse blood according to Reye (1911). The globulin solution (190 ml.) was stored in the ice chest with a thymol crystal. (Found, by micro-Kjeldahl: N, 1·00, 0·98%.)

Coupling of the azide with horse-serum globulin. To 16 ml. of the horse-serum globulin solution 10 ml. of water were added. The mixture (0·62% N) was made alkaline to phenolphthalein with 2N-NaOH, 10 ml. of dioxan were added with stirring and the resulting solution was chilled in the ice chest. The azide (300 mg.; 1 mmol.) was dissolved in 5 ml. dioxan and added slowly during 1 hr. to the globulin solution. A white precipitate formed. On addition of a further 16 ml. dioxan and 12 ml. water most of the precipitate redissolved. Throughout the experiment the reaction mixture was shaken mechanically and kept alkaline to phenolphthalein by dropwise addition of 0·1N-NaOH. At the end of the reaction (4 hr.) 9·7 ml. had been used (theory requires 10 ml.). The colloidal suspension was adjusted to pH 3·5 by addition of 2N-HCl when a white precipitate formed. After keeping at 0° for 1 hr. the mixture was centrifuged. The precipitate was suspended in 20 ml. of water and dialyzed for 3 days against running tap water. The complex was now divided into fractions soluble or insoluble at pH 9. The white sludge from the dialysis bag was made just alkaline to phenolphthalein with N-NaOH and shaken for 30 min. The suspension was centrifuged, the supernatant solution adjusted to pH 7·0 with N-HCl and stored in the ice chest with a thymol crystal. A 10 ml. portion of the cloudy solution (70 ml.) was evaporated to dryness by freeze drying under high vacuum over P_2O_5. The white, solid residue (20 mg.) was ground finely and analyzed. No methoxyl was found. Thus the complex

required is not appreciably soluble at pH 9 and was present in the precipitate.

The white precipitate from the last centrifugal treatment was dried in the same manner. The white residue contained 2·17% OCH_3, corresponding to 19·6% 7-methyl ether of bisdehydrodoisynolic residue. The ED_{50} in spayed rats was about 5 μg. using the standard technique employed at this Institute (Wilder Smith & Williams, 1947). An analysis of the original untreated horse-serum globulin solution showed no methoxyl present.

2-(6′-*Methoxy*-2′-*naphthyl*)-1:1-*dimethyl*-n-*valeric acid-globulin complex*

Chloride of 2-(6′-*methoxy*-2′-*naphthyl*)-1:1-*dimethyl*-n-*valeric acid*. The acid (1 g.; colourless crystals, m.p. 138–139°; kindly supplied by Dr Horeau) was converted into the chloride by the method of Anner *et al.* (1946); the reaction was complete after 20 hr. After one recrystallization from light petroleum (b.p. 40–60°) 810 mg. (76% of theory) of *acid chloride* were obtained in colourless needles, m.p. 63–64°. (Found: Cl, 11·7. $C_{18}H_{21}O_2Cl$ requires Cl, 11·6%.)

Azide. The acid chloride (300 mg.; 1 mmol.) was treated with NaN_3 as described above. On evaporation to dryness in N_2 at 12 mm. the *azide* was obtained as a colourless oil (300 mg.) which solidified on cooling to $-60°$ in solid CO_2. No chlorine was detectable in the flame test and the substance decomposed with evolution of gas. For characterization and identification the corresponding symmetrical disubstituted urea was prepared as follows.

NN′-*Di*-[2-(6′-*methoxy*-2′-*naphthyl*)-1:1-*dimethyl*-*butyl*]-*urea*. The acid chloride (300 mg.; 1 mmol.) was converted to the azide as described previously and the reaction mixture, containing the azide and NaCl in aqueous acetone, was refluxed on the steam bath. During 2 hr., 23 ml. of N_2 (theory 25 ml.) were evolved; the evolution of gas then ceased. The acetone was evaporated off at normal pressure and ether was added. After washing with water, the ethereal layer was dried over Na_2SO_4. On evaporation and drying under high vacuum, 250 mg. (93% of theory) of a residue were obtained, solidifying in colourless needles. After three crystallizations from light petroleum, b.p. 40–60°, 185 mg. (69% of theory) of pure *substituted urea* were obtained, m.p. 72–73°. A sample was dried for 3 hr. under high vacuum at 20°. (Found: N, 5·13. $C_{35}H_{44}O_3N_2$ requires N, 5·18%.)

Coupling of the azide with horse-serum globulin. Horse-serum globulin solution (16 ml.) was diluted with 10 ml. water and made alkaline to phenolphthalein with 2N-NaOH. After adding 10 ml. dioxan the solution was chilled in the ice chest. The azide (300 mg.; 1 mmol.) was dissolved in 5 ml. dioxan and added during 1 hr. to the globulin solution with stirring, and the resulting suspension treated exactly as described for the coupling of bisdehydrodoisynolic acid azide with globulin.

The fraction insoluble at pH 9 was freeze dried and analyzed. (Found: OCH_3, 3·57%; corresponding to 31% acid residue.) The ED_{50} was found to be about 15 μg. in spayed rats when tested by the technique used in this Institute (Wilder Smith & Williams, 1947).

SUMMARY

The coupling of the 7-methyl ether of racemic bisdehydrodoisynolic acid and of 2-(6′-methoxy-2′-naphthyl)-1:1-dimethyl-*n*-valeric acid with horse-serum globulin, with a view to the investigation of the immunological properties of the resultant complexes, has been described.

We are very grateful to the Council of the Middlesex Hospital Medical School for the provision of laboratory facilities for this work. One of us (W.H.) wishes to thank the Swiss Gnehm Stiftung for a scholarship held for a part of the period during which the research was carried out, and Prof. E. C. Dodds, F.R.S., who suggested this problem, for a grant.

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Seed Globulins of the Gramineae and Leguminosae

By C. E. DANIELSSON, *Institute of Physical Chemistry, Uppsala*

(*Received* 17 *June* 1948)

1. SEED GLOBULINS OF THE MOST COMMON SPECIES OF THE GRAMINEAE AND THEIR DIFFERENTIATION IN THE SEED

The globulins in the seeds of different species of grasses do not occur in large amounts. The following values, taken from Osborne (1909), refer to seeds of wheat: glutenin, 4·68; gliadin, 3·96; globulin, 0·62; albumin, 0·39; 'proteose', 0·21 %. It is evident that the globulins comprise only about 6 % of the total protein. This naturally causes difficulties in carrying out investigations since yields are small.

A very valuable contribution to the knowledge of the vegetable proteins was made by Quensel (1942) in his investigations of the seed globulins of barley. His method for isolating them was based on Osborne's (1909) earlier work, and made use of the solubility of globulins in dilute salt solutions, and their precipitation by ammonium sulphate or by dialysis against water.

EXPERIMENTAL AND RESULTS

General method for the preparation of globulin fractions

Since Quensel's (1942) method of preparation was followed to a large extent in these investigations, only a brief account will be given here. Quensel extracted overnight at 4° c. 800 g. of barley meal with M-NaCl, buffered with phosphate to pH 7·0. By fractional precipitation of the extract with solid $(NH_4)_2SO_4$, after clarifying by filtration, three fractions were obtained. By dissolving the precipitates in 0·2M-NaCl, pH 7·0, and subsequent dialysis of the solution in a cellophan sac against distilled water, the globulins were separated from the albumins, low-molecular and polydisperse material. In this way there could be obtained from barley seeds three fractions containing varying amounts of four well defined globulins called respectively α, β, γ and δ, in order of increasing molecular weight. These components were distributed amongst the different fractions in the following way: (1) 15%* saturation with $(NH_4)_2SO_4$, β-rich fraction; (2) 40% saturation with $(NH_4)_2SO_4$, polydisperse fraction; (3) 70% saturation with $(NH_4)_2SO_4$, α-, γ- and δ-rich fraction.

It may be noted here that the δ-component, as shown by later investigations, only appeared occasionally in the sedimentation diagrams, and then only in small concentrations.

Method of investigation

This for the most part was based on ultracentrifugation (see Svedberg & Pedersen, 1940). In the presentation of sedimentation constants, Svedberg units have been used ($1\ S = 10^{-13}$ cm.sec.$^{-1}$ dyne^{-1}). In the diagrams, the distance from the centre of rotation is plotted on the abscissa and the displacement of the scale divisions (Z) on the ordinate. To the left is the meniscus of the liquid at a distance of 5·8–6·0 cm. from the centre of the rotor, and to the right is the bottom of the cell, about 7·2 cm. from the centre. Since the displacement of the scale divisions is proportional to the concentration gradient in the cell, the area between the curve and the abscissa is proportional to the concentration. Each peak in the diagram corresponds to a protein component.

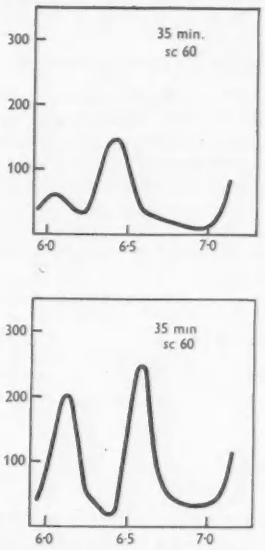

Fig. 1. Sedimentation diagrams of barley globulins. Upper: fraction obtained at 15% saturation with $(NH_4)_2SO_4$. Lower: 70% saturation. In all the sedimentation diagrams in this paper the abscissae represent distance from the centre of rotation (cm.), the ordinates represent the scale-line displacement (μ.), and sc = distance from scale (mm.). Time of sedimentation is given in min.

Quensel (1942) found the following values of the sedimentation constants for the globulin components isolated from barley: $\alpha = 2{\cdot}49\ S$, $\beta = 6{\cdot}21\ S$, $\gamma = 8{\cdot}30\ S$, $\delta = 12{\cdot}0\ S$. Although he did not succeed in isolating any of these components separately, he worked out a method for determining the molecular weights of different components in

* 100% saturation = 760 g. $(NH_4)_2SO_4$/l.

mixtures of this kind. The molecular weight is calculated from the Svedberg formula $M = \frac{RTs}{D(1 - V\rho)}$, where R = the gas constant; T = absolute temperature; V = partial specific volume; ρ = density of the solvent; M = molecular weight; s = sedimentation constant and D = diffusion constant. Taking $V = 0{\cdot}72$ and the above values for s_{20} together with the values of the diffusion constants determined by Quensel's special method ($D_\alpha = 8{\cdot}4$ and $D_\gamma = 4{\cdot}4$ in 10^{-7} c.g.s. units), $M_\alpha = 26{,}000$ and $M_\gamma = 166{,}000$. The molecular weights for β and δ were estimated from their sedimentation constants to be $M_\beta = 100{,}000$ and $M_\delta = 300{,}000$. Typical sedimentation diagrams from barley are given in Fig. 1. The α and γ components always appear at the same time in the sedimentation diagrams, as mentioned above. Quensel tried to separate these two components, but this proved impossible with his methods, which included electrophoresis and centrifugation in an air centrifuge. He points out that no genetical relation between the components could be established, and further that there was no possibility of deciding on the basis of his investigations whether or not the different components were differentiated in the seed itself. The last question has been taken for investigation in this work.

The globulins of Gramineae other than barley

Since Quensel's work only applied to barley it is of interest to see in what degree these globulin components are found in other species of the same plant family. In investigations of this point the preparation scheme of Quensel has been followed, and the results which were obtained are given in Table 1 and

Table 1. *Globulins of species of Gramineae*

Species	Components				Sedimentation constants (S)			
Hordeum vulgare	α	β	γ	δ	2·5	6·2	8·3	12·0
Secale cereale	α	—	γ	—	2·6	—	8·2	—
Triticum vulgare	α	—	γ	—	2·5	—	8·2	—
Avena sativa	α	—	γ	—	2·6	—	8·1	—
Zea Mays	α	—	γ	—	2·6	—	8·5	—
Panicum miliaceum	—	—	γ	—	—	—	8·5	—
Phleum pratense	—	—	γ	—	—	—	8·2	—
Festuca pratensis	—	—	γ	—	—	—	8·3	—
Festuca rubra	α	—	—	—	2·4	—	—	—

Fig. 2. The investigation had to be limited to the species occurring most commonly in commerce. It appears that the protein content varies greatly with the different species, and with some species is remarkably low. This is particularly true of *Phleum pratense* and the *Festuca* species, the seeds of which are very small, and seem to contain very small amounts of reserve protein. The preparation of globulins from these species is thus difficult and a negative result must not be considered as an absolute sign of their absence. Earlier work (Säverborn, Danielsson & Svedberg, 1944) has shown that malt contains the same components as barley, and the sedimentation diagrams from barley and malt are identical.

Quensel's investigations showed that the sedimentation constant of the γ component depends on concentration, i.e. $s = 8{\cdot}30 - 0{\cdot}6\,c$, where c is the percentage concentration and 8·30 is the sedimentation constant at infinite dilution ($c = 0$). The values in Table 1 have been corrected with the help of this equation. The concentrations have been obtained partly by measurement of the refractive index of the solution, and partly by calculation from the sedimentation diagram.

Hordeum vulgare (barley) is unique in this family, since it alone contains the β component, which occurs in high concentration. Barley is also the only species which contains the δ component, although only in low concentration.

The β component has some very interesting properties, which may possibly be connected with certain phenomena in the brewing of beer. The relations are not altogether clear, but it seems possible that the β component is part of the precipitate which is obtained on storing beer in the cold. Investigations at this Institute show that in this precipitate the β component may be combined with tannic acid. The β component is very stable towards many agents such as heat and enzymes, and it has been possible to show (Säverborn *et al.* 1944) that it survives the mashing process in the production of beer which causes degradation of the other proteins. The β component is distinguished from the other components by its content of 1·96 % S, compared with 1·65 % for the α and γ components. These proteins thus differ not only in molecular weight but also in chemical constitution. This partly explains the large differences on salting out with ammonium sulphate.

If these extra components in barley are disregarded, the different species within the family seem to have, to a large extent, the same seed globulins. It is to be noticed that the γ component is predominant in the species that have little reserve protein, for example, *Phleum pratense* and *Festuca pratensis*. This is understandable in the light of our experiments, when it was found to occur only in the embryo: a very small seed with little reserve protein would thus be expected to contain mainly the γ component.

Differentiation of the components in the seed

Experiments with wheat. The question of the differentiation of the globulin components defined by Quensel (1942) was partly solved by the work on wheat (Säverborn *et al.* 1944). The separation of the components was made possible by the fact that, in the milling of wheat, one can separate the embryo from the rest of the seed.

In the commercial milling of wheat a number of different fractions is obtained. Only the most interesting fractions have been investigated here. The

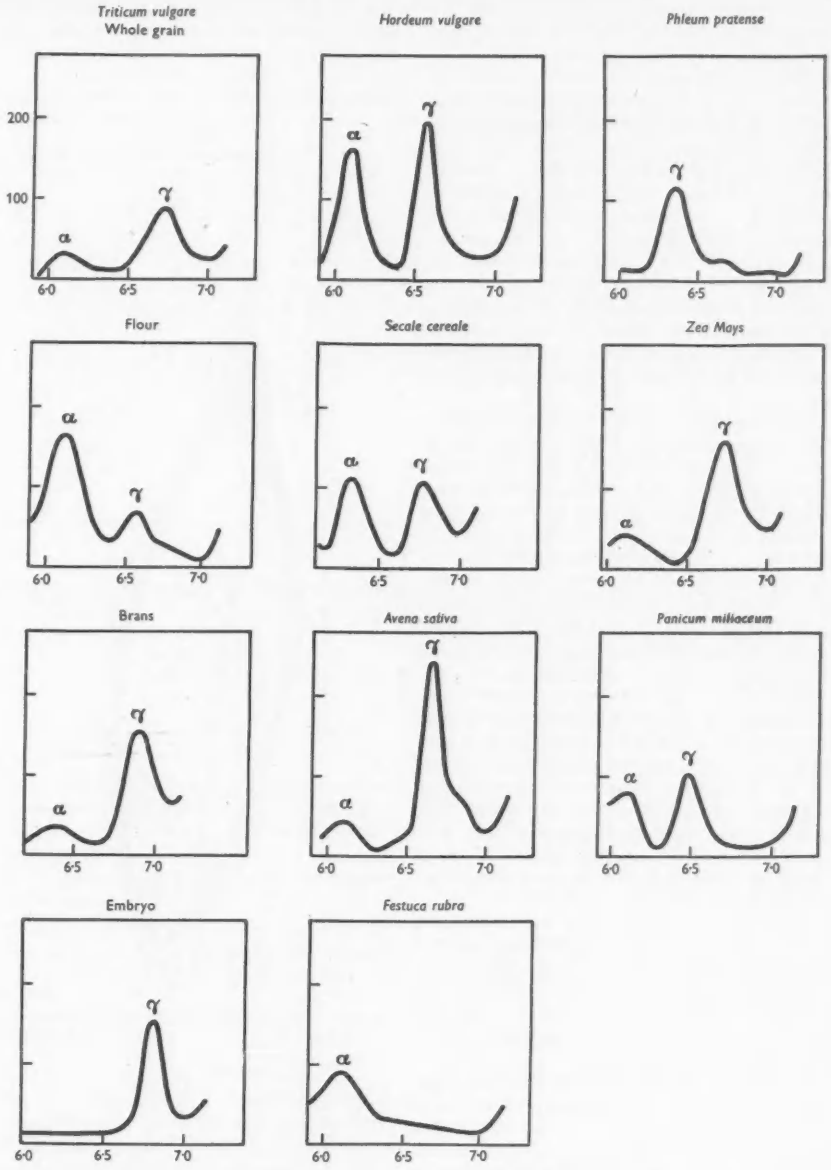

Fig. 2. Sedimentation diagrams showing the composition of globulin solutions prepared by extraction of seeds from different species of the Gramineae. The four figures to the left refer to different fractions of seeds from wheat. Ordinates and abscissae as in top left.

results obtained earlier from investigations on the whole grain were compared with those obtained by the same method on the following fractions: (*a*) bran fraction; (*b*) embryo fraction; (*c*) flour fraction with husk and embryo removed.

The sedimentation diagrams of the globulins obtained from the various fractions were practically identical with those from barley, with the important exception that the β component was absent. Exactly the same sedimentation constants were obtained. Only in respect of the concentrations of the α and γ components could a difference be observed, as can be seen from Fig. 2. The γ component is obtained completely pure from the embryo fraction, while the flour fraction contains considerably more α than γ. Finally, the bran fraction contains γ in much higher concentrations than α. This fraction contains the so-called aleurone layer, which is particularly rich in proteins.

Experiments with barley. In barley, the embryo cannot be separated directly by milling, but was obtained manually by excision with a razor blade. The two fractions (embryo and residual fraction) were investigated by the same method as before, using 100 g. of embryo and 200 g. of residual fraction for each experiment. The result obtained was in complete agreement with the above-mentioned experiments with wheat seed. The sedimentation constants were naturally identical for both barley and wheat, and equal to those obtained by Quensel (1942) for solutions containing both components.

For barley, the α component occurs entirely in the residual fraction except for a low concentration in the embryo where it is present probably as a contaminant, being introduced along with the residual fraction which adheres to the embryo when the latter is removed from the seed manually. This finding is in accord with the observation that with wheat, as a result of the nearly perfect isolation of the embryo by the milling process, the α component is found only in the residual fraction.

The γ component occurs only in the embryo fraction of both wheat and barley, being isolated completely pure from wheat and only slightly contaminated with α, as mentioned above, in the barley preparation. Finally, the β component has been found only in barley seeds where it is present solely in the residual fraction.

The distribution of the different globulin components in the seeds of wheat and barley is shown in Table 2 and Fig. 3. It is of interest that the protein of highest molecular weight is in the embryo, the most vital part of the seed. It is perhaps premature to draw conclusions concerning the utilization of the proteins in the germination itself and in the formation of the young plant, but it is conceivable that the different components have different functions in germination. The latter causes a large diminution in the concentrations of these components, and it is reasonable to suppose that they are directly broken down to smaller units, which can be easily utilized by the young plant.

Table 2. *Distribution of globulins within the wheat and barley seed*

	Embryo fraction			Residual fraction		
	α	β	γ	α	β	γ
Wheat	−	−	+ + +	+ + +	−	+
Barley	+	−	+ + +	+ + +	+ + +	−

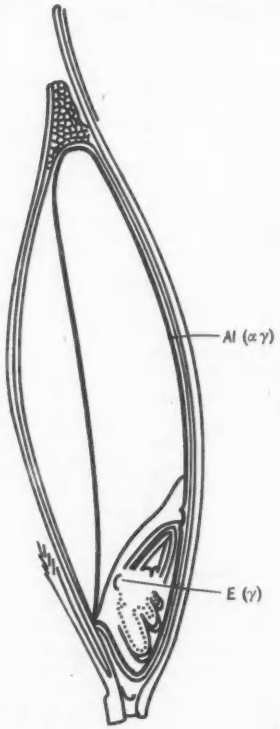

Fig. 3. Diagram showing the localization of the α and the γ components in seeds of wheat. *Al*, aleurone grains; *E*, embryo.

Whether there exists a biogenetical relationship between the globulin components described here is very difficult to decide. Many attempts have been made to degrade in different ways the purified γ component from wheat embryo in order to find out whether the α component can be obtained as an intermediate product, but without success. Ultrasonic vibration had no influence on the γ component, whilst among the proteolytic enzymes, papain

caused a very slow but continuous degradation under optimal conditions, and the process could easily be followed in the ultracentrifuge. No well-defined high-molecular intermediate products have been found under any circumstances, leaving the question of a possible relationship between the components unsettled.

(1937). Fig. 4 shows the results of the diffusion measurements for the pure γ component from wheat embryo. The diffusion constant has been calculated by two methods, which give the values D_m and D_A. For a substance having ideal diffusion both methods must of course give the same value for D. The values in Table 3 have been calculated from the curves in

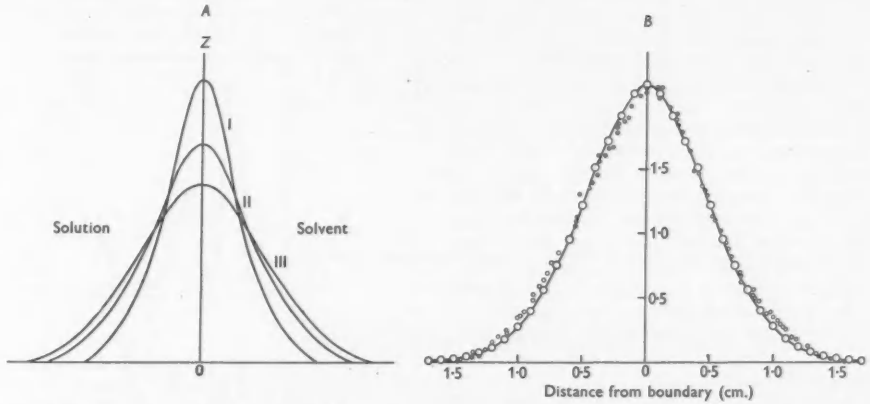

Fig. 4. Diffusion diagrams of the γ component of wheat embryo. *A*, the curves marked I, II and III refer to exposures taken 29, 49 and 73 hr. after the beginning of the diffusion process and show the concentration gradient as a function of the distance from the originally sharp boundary between solvent and solution (0). *B*, the diffusion curves I, II and III from *A* have been transformed to normal co-ordinates, marked by small circles. The larger circles and the curve correspond to an ideal diffusion curve. Z = displacement of scale divisions.

Determinations of the molecular weights of the α and γ components

The methods described above for the isolation of the α and γ components have been used for the preparation of pure components for diffusion measurements. In this way α has been prepared from barley after removal of the embryo, while the γ component has been obtained from the wheat embryo.

In the determination of the molecular weight of the γ component, the concentration dependence of the sedimentation constant must be taken into consideration. In this investigation the value $s_{20} = 8{\cdot}7\ S$ at infinite dilution was obtained for the γ component of wheat embryo, whereas Quensel (1942) found the value 8·30 *S*, or about 5% less for γ from barley. However, in Quensel's experiments, the sedimentation of the γ component was disturbed by the presence of α, the contribution of which to the viscosity of the medium was not known and was accordingly omitted from the calculations. The sedimentation constant reported here for wheat γ is presumably correct since the preparation was pure. The apparent difference, therefore, in s_{20} for γ globulin from wheat and barley may be merely a result of experimental inaccuracy.

The diffusion measurements have been made with the help of the scale method worked out by Lamm

Fig. 4. There is a steady decrease with time in the values for both D_m and D_A, which has persisted in later experiments. $D = 3{\cdot}6 \times 10^{-7}$ c.g.s. is taken as the average.

Table 3. *D_{20} of the γ component of wheat embryo*

(D is given in units of 10^{-7} c.g.s. at 20°.)

	Diffusion time (hr.)					
	29	36	49	57	73	Average
D_m	3·72	3·71	3·52	3·41	3·44	3·56
D_A	3·76	3·71	3·57	3·58	3·46	3·62

Table 4. *D_{20} of the α component of barley*

(D is given in units of 10^{-7} c.g.s. at 20°.)

	Diffusion time (hr.)					
	9	22	35	45	59	Average
D_m	7·99	7·64	7·64	6·81	7·08	7·43
D_A	7·78	7·35	7·34	6·79	7·10	7·27

From the values $s_{20} = 8{\cdot}7\ S$, $D = 3{\cdot}6 \times 10^{-7}$ c.g.s., $V = 0{\cdot}72$ (Quensel, 1942) and $\rho = 0{\cdot}9982$, the molecular weight $M_\gamma = 210{,}000$ is obtained. This exceeds

Quensel's value by 28 %, the difference being due to the fact that we used the isolated and completely pure γ component, which led to a greater accuracy of measurement. The values of D for the α component of barley are given in Table 4. The agreement between D_m and D_A may be considered satisfactory. By using $s_{20} = 2{\cdot}5\ S$, $D = 7{\cdot}4 \times 10^{-7}$ c.g.s., $V = 0{\cdot}72$ (Quensel) and $\rho = 0{\cdot}9982$ one gets $M_\alpha = 29{\cdot}000$, a value which thus lies very near to that found by Quensel.

2. SEED GLOBULINS OF THE LEGUMINOSAE

The seeds of leguminous plants are very rich in proteins, containing 30–50 % total protein compared with 7–10 % for members of the Gramineae. It was assumed, therefore, that these globulins would be easier to prepare than those of the Gramineae, and this proved to be so.

Careful investigations of globulins from peas have been carried out by Osborne (1898). Apart from elementary analyses, Osborne studied chiefly the solubility and coagulation properties of the globulins. From these latter studies Osborne was able to isolate three globulin fractions from peas: (1) legumin, does not coagulate on heating to 100°; (2) vicilin, coagulates on heating to 95–100°, soluble in more dilute salt solutions than legumin; (3) legumelin, occupies an intermediate position with respect to globulins and albumins and is partly precipitated on dialysis.

In the experiments which will be described, Osborne's method of preparation has mainly been used, viz. precipitation with ammonium sulphate and dialysis. Our preparations, therefore, should be very similar to those which Osborne analyzed. The same scheme of preparation has been employed here as in the earlier experiments on the Gramineae; seeds of *Pisum sativum* have been mainly used.

Experimental and results

The sodium chloride extract of the ground seeds was at first precipitated with solid ammonium sulphate at three different degrees of saturation, 15, 40 and 70 %. Ultracentrifuge diagrams of the three fractions are all similar, and indicate two components (Fig. 5), with sedimentation constants about 7·5 and 12·5 S. Since the different fractions contained the same globulin components in about the same concentration the extract was subsequently precipitated directly at 70 % saturation. The following scheme of preparation has been used.

Finely ground seeds are extracted for 1 night at 4° with 10 times their weight of M-NaCl, buffered with phosphate to pH 7. The solution is then centrifuged, and the extract filtered clear. Solid $(NH_4)_2SO_4$ is added with stirring to 70 % saturation (532 g. of salt/l.) and the precipitate centrifuged down. It is dissolved in 0·2 M-NaCl, buffered to pH 7. After filtration, the solution obtained is dialyzed against running water in a cellophan sac. After 12–24 hr. the globulins have precipitated. They are recovered by centrifuging, the precipitate is redissolved, and the solution dialyzed once more until precipitation has occurred. The precipitate obtained from the second dialysis is dried *in vacuo* over $CaSO_4$. In this way, a yellowish white powder is obtained which is very readily soluble in salt solutions at pH 7. A buffer with the following composition has been used here: 0·2 M-NaCl; 0·03 M-Na_2HPO_4; 0·02 M-NaH_2PO_4; pH 7·0. This buffer will be referred to as the standard buffer. The dried preparation can be stored indefinitely, and the solubility does not change with time (no change has been noticed during 1 year).

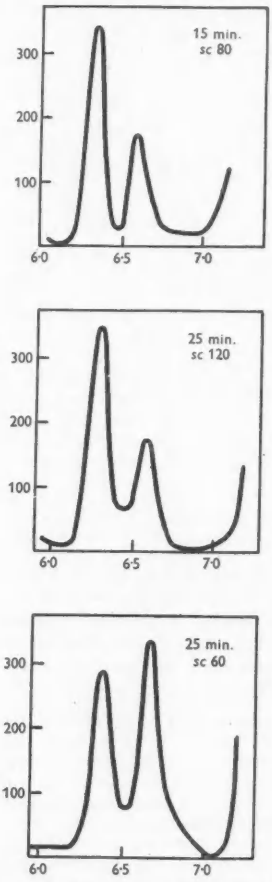

Fig. 5. Sedimentation diagrams showing the globulin composition in seeds of *Pisum sativum*: upper, 15 % saturation with $(NH_4)_2SO_4$; centre, 40 %; lower, 70 %.

The yield varies of course from preparation to preparation, depending on how completely the substance precipitates during the two dialyses. Usually a yield of 1·5–2·0% of the pure components is obtained. Peas contain 21·5% total protein (Czapek, 1905). The globulins, therefore, comprise about 10% of the total protein.

mentation diagram of the 70% saturation fraction from peas.

In order to obtain more reliable values of the sedimentation constants, the centrifugations have been carried out at different concentrations. The results are given in Fig. 7. Thus, for the component with the higher sedimentation constant, no de-

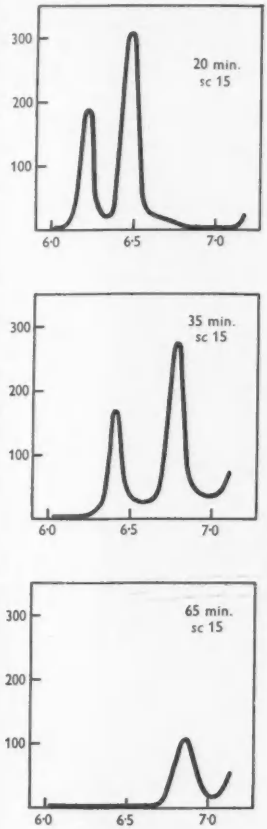

Fig. 6.

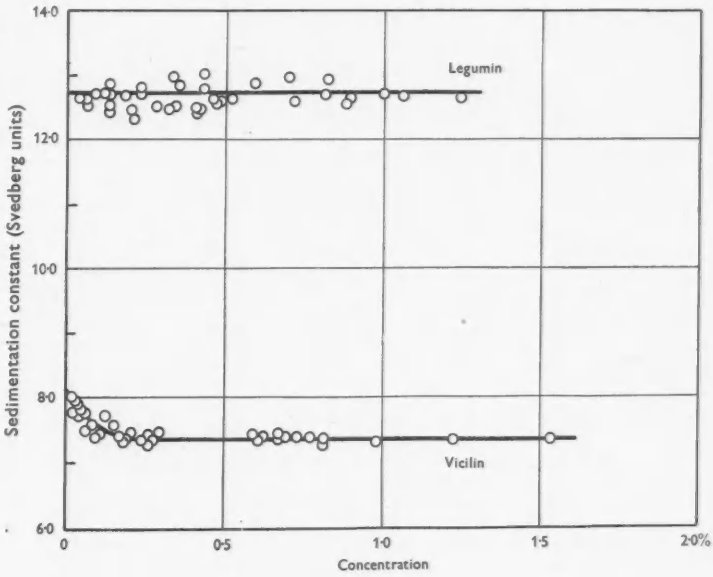

Fig. 7.

Fig. 6. Sedimentation diagrams of the fraction obtained at 70% saturation with $(NH_4)_2SO_4$ (*Pisum sativum*). The diagrams correspond to exposures taken 20, 35 and 65 min. after the beginning of sedimentation at 65,000 r.p.m.

Fig. 7. The relation between the sedimentation constants and the concentrations for the two globulin components in seeds from pea.

Investigation of pea globulins in the ultracentrifuge

As shown earlier (Fig. 5), two components are obtained on ultracentrifuging the globulin fractions prepared as above. Fig. 6 shows a typical sedi-

pendence of the sedimentation constant on concentration can be established, indicating that the particles are probably not highly asymmetrical. A value of the sedimentation constant for this component has been obtained by taking the average of the values given graphically in Fig. 7, the average of

forty values being $s_{20} = 12{\cdot}64\ S$. The sedimentation constant for the other component from peas shows, however, a distinct dependence on concentration. The sedimentation constant is usually given for infinite dilution, i.e. for zero concentration, and from Fig. 7 the value $s_{20} = 8{\cdot}10\ S$ is obtained.

Attempts to separate the components

In the experiments described above both components have appeared at the same time in the diagrams. Experiments to isolate each component separately have been carried out in order that molecular weight determinations could be made.

buffer and precipitated with 11·0 g. of $(NH_4)_2SO_4$. The precipitate was centrifuged off and dissolved in 10 ml. of standard buffer, after which the solution was dialyzed for 2 days. The precipitate obtained was dissolved in 10 ml. of standard buffer. The solution was designated *C* and investigated in the ultracentrifuge.

The results of these precipitation experiments are seen in Fig. 8, which shows that by means of fractional precipitation it is possible to obtain the heavier globulin component relatively pure. The method is not, however, sufficiently effective. It has proved to be very difficult to remove the last traces of the lighter component, even if the method of separation described above is repeated.

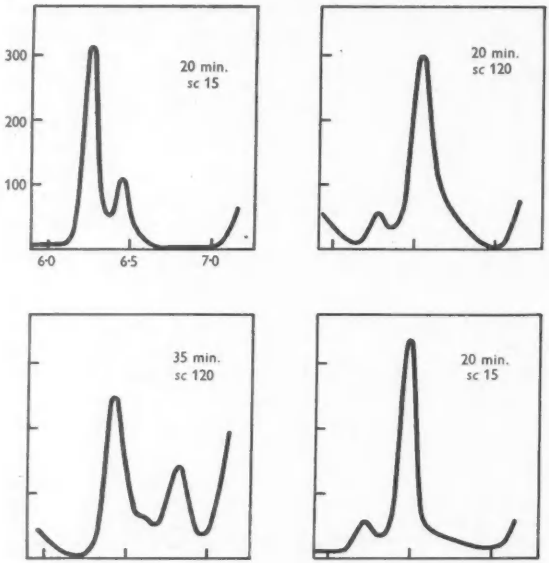

Fig. 8. Sedimentation diagrams after fractional precipitation of vicilin and legumin with $(NH_4)_2SO_4$. Top left, initial solution; top right, solution *A*; bottom left, solution *B*; bottom right, solution *C* (see text). Ordinates and abscissae as in top left.

Fractional precipitation with ammonium sulphate. The following experiment was carried out: 12·0 g. of $(NH_4)_2SO_4$ were added to 25 ml. of 0·6% pea-globulin solution in the standard buffer, with stirring. The precipitate obtained was dissolved in 8·0 ml. of standard buffer (solution *A*). To the centrifugate obtained above were added an additional 3 g. of $(NH_4)_2SO_4$ after which the precipitate obtained was centrifuged down and dissolved in 10 ml. of standard buffer (solution *B*). On further addition of salt no more precipitation was obtained.

Both the solutions, *A* and *B*, were dialyzed against distilled water for 2 days at 4°. In this way the globulins precipitated quantitatively. After centrifuging down, the precipitates were dissolved in 10 ml. of standard buffer, and investigated in the ultracentrifuge, whilst the supernatant fluid from *A* (9 ml.) was diluted to 25 ml. with standard

Separation by means of dilution with water at 0°. The behaviour of solutions of pea globulin in standard buffer is strongly dependent on temperature. On cooling down to 0° a milk-white suspension is obtained after about an hour, and if the solution is allowed to stand for a day or two at this temperature, a dense precipitate is obtained on the bottom of the vessel. Preliminary experiments showed that the substance precipitated in this way contained a relatively higher concentration of the heavier component than the original solution. A method of separation based on the above-mentioned properties seemed possible. However, no satisfactory results were obtained merely by cooling the solutions, but instead the solutions were diluted with water at 0°: 10 ml. of 1% pea globulin in standard buffer were cooled to 0° and diluted with distilled water at 0°. At a total volume of 35 ml. (25 ml. of water had been added)

a dense white precipitate was suddenly obtained. An additional 65 ml. of water were added, after which the precipitate was centrifuged down at 0° in a refrigerator. Two fractions were obtained in this way: (*A*) The supernatant: this was dialyzed against water in a cellophan sac at 4°. After 24 hr. the precipitate obtained was dissolved in 10 ml. of standard buffer and investigated in the ultracentrifuge. (*B*) The precipitate: this was dissolved in 10 ml. of standard buffer, and the precipitation repeated by the addition of 90 ml. of water at 0°. This was repeated until the substance had been precipitated a total of five times. Finally, the precipitate was dissolved in 10 ml. of standard buffer and investigated in the ultracentrifuge.

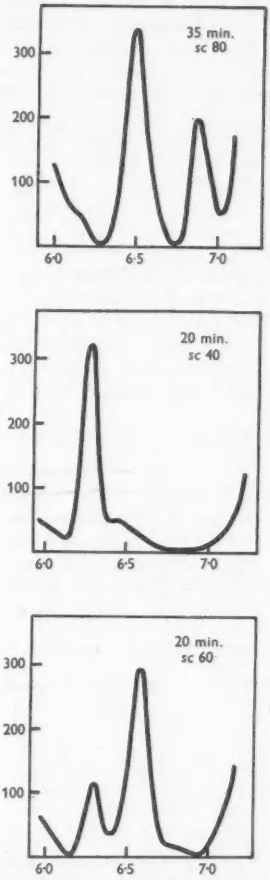

Fig. 9. Sedimentation diagrams after separation of vicilin and legumin by means of dilution at 0°. Upper, initial solution; centre, supernatant; lower, precipitate (see text).

The results of the above precipitation experiments are shown in Fig. 9. The heavier component precipitates to a considerably greater extent than the lighter on dilution at a low temperature. The isolation of the two components is not, however, completely satisfactory if it is desired to prepare solutions for diffusion experiments, and other methods must be employed.

Separation by changing the pH. Separation experiments described earlier have made possible the development of a new, effective method of separation. In a solution containing mainly the heavier component, prepared by fractional precipitation with $(NH_4)_2SO_4$ (see Fig. 8), the pH was slowly lowered. At pH 4·5–5 a precipitate was obtained

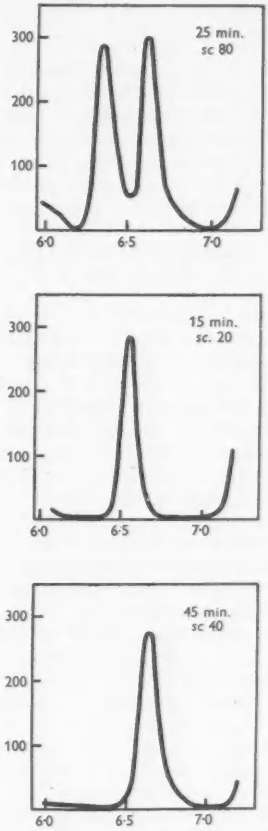

Fig. 10. Sedimentation diagrams after separation of vicilin and legumin by means of pH change. Upper, initial solution; centre, precipitate at pH 4·5 (legumin); lower, supernatant at 4·5 (vicilin) (see p. 396 of text).

which was soluble on both sides of this precipitation range. In a solution containing the lighter component, prepared by dilution with water in the cold (see Fig. 9), no precipitation occurs at any pH (only the range from pH 3 to 10 has been

investigated here in view of the risk of denaturation). This property has given rise to the most effective method so far for the separation of the two components. A dried preparation, consisting of a mixture of approximately equal portions of the two components, is treated with a large excess of buffer of pH 4·5, with stirring. Acetate buffers containing 0·2 M-NaCl have been used. During this procedure the lighter component dissolves, while the other does not. After 24 hr. the heavier component is centrifuged off and dissolved in standard buffer. This solution is dialyzed against water. The centrifugate from the above centrifugation contains the lighter component, and this solution is also dialyzed. After 1–2 days all the globulin has precipitated, and the two precipitates are dried in the cold. On ultracentrifugation of the fractions so obtained the diagrams of Fig. 10 are obtained.

This method of separation, based on isoelectric precipitation of the heavier component, is clearly the most effective of the three methods described here. By repeated use of this method the two components have been completely separated from each other, which makes possible molecular weight determinations of both globulin components.

Comparison with Osborne's globulin components

By isoelectric precipitation two solutions were prepared, one containing the lighter component and the other the heavier. Coagulation experiments were carried out on these fractions, in order, if possible, to obtain some connexion between these two components and the globulin components defined by Osborne (1898). On boiling a solution containing the heavier component, no precipitate was obtained, i.e. the substance does not coagulate on warming to 100°. This component should, therefore, consist of legumin, according to Osborne's definition. On boiling, the component is of course destroyed, which is also shown by ultracentrifugation.

On warming a solution containing the lighter component, precipitation is obtained at 95°, and, on boiling, all the protein precipitates. The lighter globulin component consists, therefore, of vicilin. In what follows the two components will be given these names. Legumin is the component which has a sedimentation constant of 12·64 S, while vicilin has a sedimentation constant of 8·10 S at infinite dilution.

As mentioned earlier, Osborne has pointed out that vicilin is soluble in more dilute salt solutions than legumin. This is also clearly seen from the separation experiments by dilution with water at 0°. It would seem to be proved by this that the globulin components isolated by Osborne, legumin and vicilin, are identical with the globulins from peas described in this paper. Nothing corresponding to the legumelin defined by Osborne has, however, been detectable on ultracentrifugation.

Determination of the molecular weight of vicilin

The initial solution for determinations of D and V of vicilin was prepared according to the method described earlier for the separation of vicilin from legumin by isoelectric precipitation of the legumin. This method was applied several times so that the vicilin solution obtained was completely freed from legumin, as was established by ultracentrifugation. D was determined according to Lamm's (1937) scale method in a stainless-steel cell designed by Claesson (1946), but somewhat modified. The values of D_m and D_A obtained after different diffusion times are given in Table 5. The agreement between the two

Table 5. *Diffusion constant of vicilin*

(D is given in units of 10^{-7} c.g.s. at 20°.)

	Diffusion time (hr.)				
	23	28	32	46	Average
D_m	4·44	4·18	4·22	4·30	4·29
D_A	4·31	4·20	4·18	4·17	4·22

different methods of calculation is good. Taking an average value of $D = 4{\cdot}26 \times 10^{-7}$ c.g.s., and an experimentally determined value of $V_{20} = 0{\cdot}752$, the molecular weight of vicilin, using the value of the sedimentation constant found earlier, $s_{20} = 8{\cdot}10$ S, and $\rho = 0{\cdot}9982$, is found to be $M = 186{,}000$.

Determination of the molecular weight of legumin

The initial solution for the determination of D and V in the case of legumin had been completely freed from vicilin by repeated isoelectric precipitation. D was determined in the same cell and in exactly the same way as that of vicilin. The results are given in Table 6. In this case also the agreement

Table 6. *Diffusion constant of legumin*

(D is given in units of 10^{-7} c.g.s. at 20°.)

	Diffusion time (hr.)					
	23	25	28	45	48	Average
D_m	3·62	3·57	3·52	3·32	3·42	3·50
D_A	3·52	3·39	3·45	3·53	3·53	3·48

between the values of D_m and D_A is very good, indicating a high degree of monodispersity. For the calculation of the molecular weight of the legumin the values used were: $D = 3{\cdot}49 \times 10^{-7}$ c.g.s. and $s_{20} = 12{\cdot}64$ S. V was determined, as in the previous case, by Prof. C. Drucker, who obtained the value $V_{20} = 0{\cdot}735$. With the value $\rho = 0{\cdot}9982$, the molecular weight for legumin is found to be $M = 331{,}000$.

Occurrence of vicilin and legumin in different species of the Leguminosae

The experiments described earlier to isolate seed globulins from different species of the Gramineae supported the working hypothesis that closely related plants contain the same types of protein. A further confirmation of this hypothesis seems to be given by the following investigations on different species of the Leguminosae.

Experiments to isolate the globulins from seeds of different Leguminosae have been carried out in exactly the same way for all the species. Owing to the difficulties in obtaining a sufficient amount of seed of some of the species, the amount of initial substance has varied between 3 and 10 g. The proportion of amount of extraction buffer to amount of seed has been kept constant throughout. The scheme of preparation has been the same as in the experiments described earlier for the isolation of vicilin and legumin from peas, *Pisum sativum*, whereby a solution containing both components is obtained.

Table 7. *Sedimentation constants of the globulin components of various Leguminosae*

(Results in Svedberg units.)

Acacia alata	1·25	7·90	11·63	—
A. decipiens	—	8·02	12·70	—
A. Farnesiana	—	8·04	—	—
A. longifolia	2·72	7·59	—	18·04
A. penninervis	1·46	7·39	—	18·75
A. saligna	—	7·76	13·67	—
A. verticillata	—	7·77	—	—
Arachis hypogaea	1·93	8·40	13·05	—
Astragalus galegiformis	—	8·33	13·17	—
Cytisus Laburnum	—	8·08	14·02	—
C. supinus	1·84	8·03	13·38	—
Dolichos Lablab	—	7·33	11·66	—
Ervum Lens	—	7·25	13·18	—
Genista tinctoria	—	8·54	13·34	20·31
Glycine Soja	—	7·97	13·06	—
Lathyrus Clymenum	—	7·55	13·00	—
L. odoratus	—	7·64	12·00	—
L. sativus	—	7·46	13·04	—
L. silvestris	—	7·48	12·97	—
Lotus Tetragonolobus	—	8·32	13·07	—
Lupinus albus	—	8·24	12·29	—
L. angustifolius	—	8·20	13·05	—
L. luteus	—	8·30	11·53	—
L. polyphyllus	—	8·69	12·20	—
Medicago sativa	—	6·77	11·41	—
Phaseolus coccineus	4·29	7·39	12·16	—
P. nanus	—	6·55	10·10	—
P. vulgaris	4·87	7·26	11·02	—
Pisum sativum	—	8·10	12·64	—
Trifolium hybridum	—	7·66	12·90	—
T. pratense	—	7·69	11·22	—
T. repens	—	7·27	—	18·20
Vicia Faba	—	7·12	11·80	—
V. sativa	—	7·09	11·91	—

As follows from Table 7 and Fig. 11, vicilin and legumin occur in the seeds of practically all the species investigated of the Leguminosae. The most important exceptions are a few *Acacia* species (*A. longifolia*, *A. penninervis*, and *A. verticillata*). In these species, legumin is absent, while in the other four *Acacia* species it has been possible to isolate legumin, even although in rather small quantities. On comparing Acaciae with the other species of the Leguminosae it is seen that the former hold a unique position in this family; they are, in fact, sometimes considered as a subfamily within the Leguminosae, and this may be justified to a certain extent by the protein composition of the seeds. Furthermore, it has not been possible to isolate legumin from the seeds of *Trifolium repens*. However, this apparent absence of legumin may simply result from difficulties in preparation, since the various *Trifolium* species are relatively poor in proteins.

It is seen from Table 7 that in some cases globulin components have been isolated with a sedimentation constant of $s_{20} \approx 2\ S$. These components probably constitute a degradation product of legumin. According to investigations, as yet unpublished, a component of globulin nature with a sedimentation constant of $1{\cdot}9\ S$ is obtained by dissolving legumin in a buffer of pH 3. The same component is obtained from legumin by protracted heating at 100°. In some cases, a component of $s_{20} \approx 18\ S$ has also been obtained, though always in very low concentration; it is found in *Pisum sativum*, but it has not been possible to separate it from vicilin and legumin. Whether it exists in the seeds, or results from complex formation between vicilin or legumin, is not yet clear. Experiments to answer this question seem, however, to indicate the latter possibility.

It is also interesting to compare the relative concentrations of vicilin and legumin in the different species. Closely related species have approximately the same relative concentrations of the two components. In the various *Lathyrus* species, for example, legumin always predominates, as is also the case for the two *Vicia* species investigated. These two genera are, of course, botanically very near to each other. In the various *Lupinus* species it is the vicilin which is in greater concentration, and this also applies to the *Phaseolus* and *Trifolium* species.

If one specially considers *Vicia sativa* it is seen from the sedimentation diagram that vicilin is found in somewhat lower concentration than legumin. This is contrary to the conception of Osborne & Campbell (1898), according to whom vicilin should be completely absent from this species.

In order to obtain more accurate values of the relative concentrations of vicilin and legumin, a new method is being developed for determining the concentrations of these components in solutions containing both, based on the fact that legumin contains about five times more tryptophan than vicilin. By measuring the absorption in the ultraviolet, the

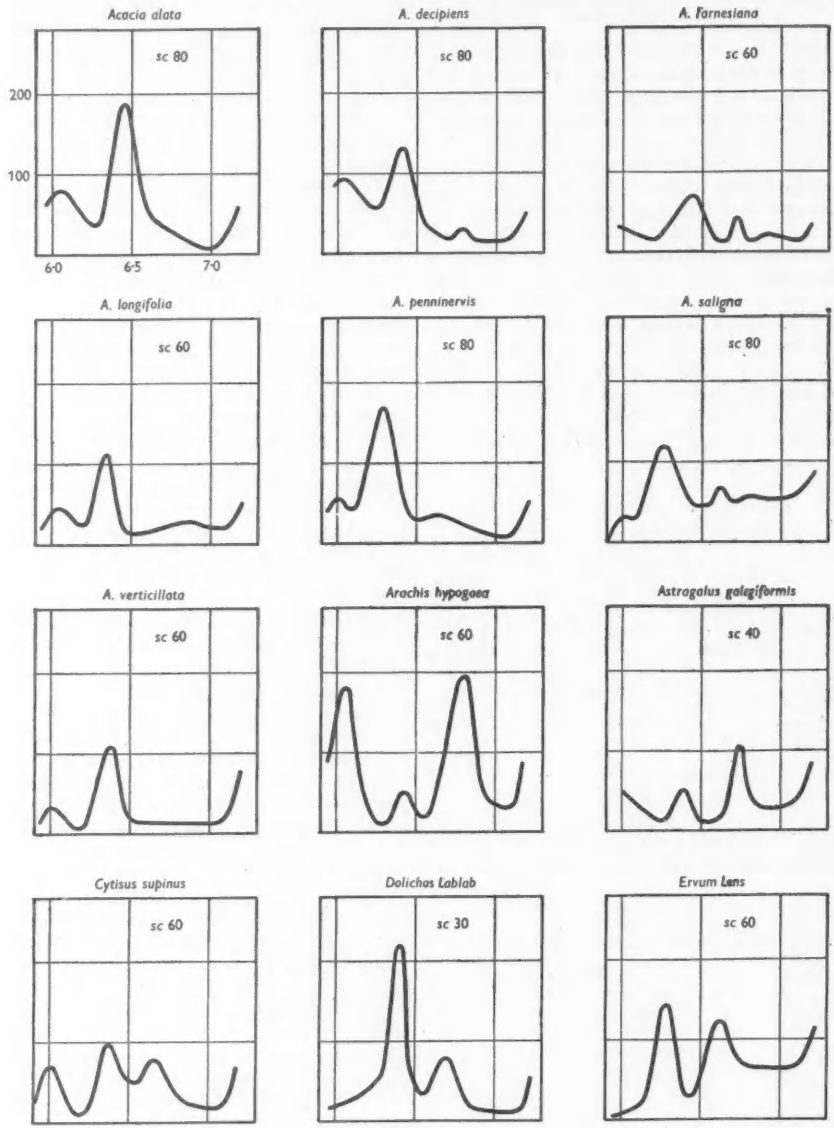

Fig. 11*a*.

Fig. 11 *a–c*. Sedimentation diagrams showing the composition of globulin solutions prepared by extraction of seeds from different species of the Leguminosae. Ordinates and abscissae as in top left.

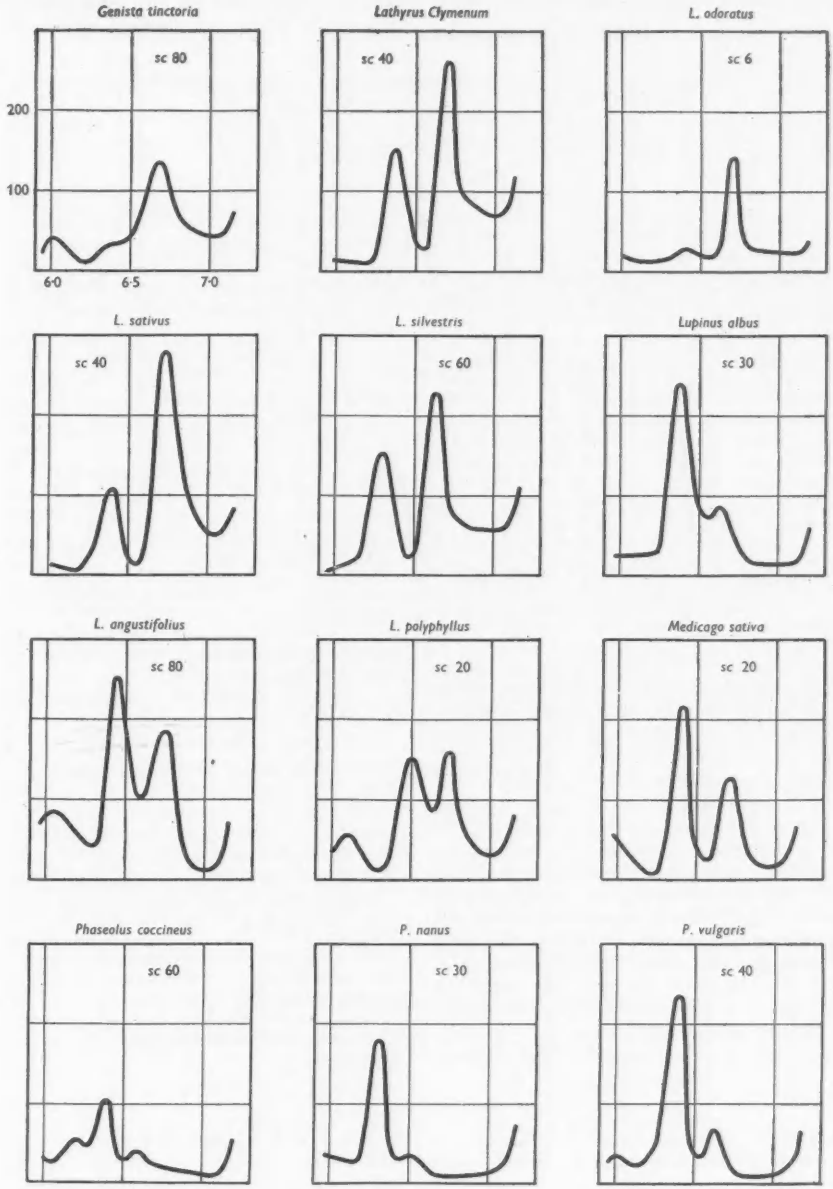

Fig. 11 *b*. For legend see p. 398.

relative concentrations of vicilin and legumin can be determined. The results will be published in a later paper.

components have been isolated. Different methods have been developed for the separation of these components, which are identical with vicilin and

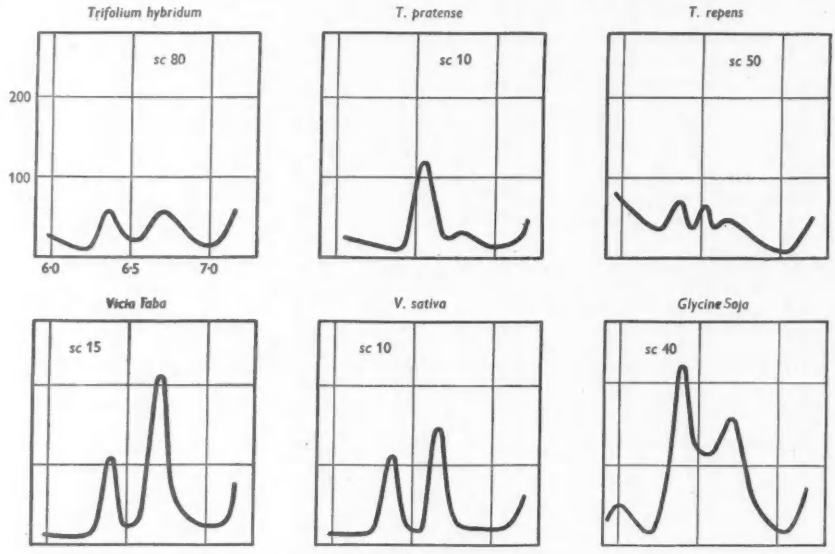

Fig. 11 *c*. For legend see p. 398.

SUMMARY

1. The seed globulins of nine different species in the family Gramineae have been investigated. Two well defined globulin components, α and γ, were isolated. The α component was found in six and the γ component in eight species. Seeds of barley contain two other components, β and δ, which were not found in any other species of this family.

2. These components occur in different parts of the seed itself. Investigations on different fractions from seeds of wheat and barley have shown that the γ component alone occurs in the embryo fraction and the α component in the residual fraction. The husk fraction contains γ in higher concentration than α.

3. Determinations of the molecular weight of the purified components have given the values $M = 29{,}000$ for α and $M = 210{,}000$ for γ.

4. From the pea, *Pisum sativum*, two globulin legumin, first defined and investigated by Osborne (1898, 1909).

5. The molecular weight of vicilin was found to be 186,000, and that of legumin 331,000.

6. The seed globulins from thirty four different species in the family Leguminosae have been investigated. Vicilin and legumin were found in all, except in some *Acacia* species and in *Trifolium repens*. In a few cases degradation products and complex compounds of these components have been isolated.

7. These investigations seem to confirm the hypothesis that closely related plants contain the same seed globulins.

The author wishes to thank Prof. Svedberg for the great and stimulating interest which he has shown in this work, and for the privilege of working in his laboratory. The expenses connected with these investigations were defrayed by the AB. Stockholms Bryggerier and the Rockefeller Foundation.

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Carotenoids of Loquat (*Eriobotrya japonica* Lindl.)

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The pigments of loquat (*Eriobotrya japonica*) do not appear to have been investigated heretofore. Loquat is a valued subtropical fruit and is grown abundantly in Northern India either from seed or from grafts. It is yellow or orange, 3–7 cm. long with a thin skin and having one to five (commonly three) seeds separated by membranous dissepiments. The fruit is pear shaped and ripens towards the end of the cold season.

After saponification of the fruit pulp, a colorimetric analysis of the total extract gave gross carotenoid values which varied from 5·1 to 56·5 μg./g. After a chromatographic separation, the true β-carotene content was found to be only 36–60% of the total pigments. From the carotene fraction, three other pigments besides β-carotene were isolated, one of which was cryptoxanthin and the other two were stereoisomers of β-carotene, viz. neo-β-carotene U (adsorbed just above the β-carotene zone) and neo-β-carotene B (adsorbed below the β-carotene zone). The hypophasic pigments have all been classed as xanthophylls other than cryptoxanthin.

EXPERIMENTAL

An accurately weighed amount of the pulp of the fresh fruit (5–6 g.) was thoroughly ground with chemically pure white sand under methanol. The ground material was quantitatively transferred to a digestion flask, and saponified in the cold with 100–125 ml. of concentrated methanolic KOH with continuous agitation to facilitate thorough digestion. The saponified mixture was filtered, and the residue extracted alternately with light petroleum (b.p. 40–60°) and methanol till the washings were colourless. Distilled water (100 ml.) was then added to the combined light petroleum and methanol fractions in a separating funnel and shaken gently. After complete separation of the two layers the methanolic solution was drawn off and treated with a fresh portion of light petroleum in a separating funnel. This process was continued until all the carotenoid pigments were transferred to light petroleum. The combined light petroleum fractions were washed free from soaps and alkali with distilled water. The first washings were re-extracted with light petroleum and returned to the principal extract. Xanthophylls were separated by repeated extractions with 90% (v/v) aqueous methanol. The methanol fraction was distilled under reduced pressure in an atmosphere of N_2. The residual pigment mixture was taken up in light petroleum and the amount present was determined colorimetrically. The epiphasic pigments were washed with distilled water to remove the last traces of methanol and dried over anhydrous Na_2SO_4. The extract was concentrated to 8–10 ml. under reduced pressure in the presence of N_2. A few crystals of quinol were added before concentration to prevent oxidation. The solution containing the pigments was passed through an adsorption column of activated alumina (obtained from Scientific Supplies (Bengal) Co., Ltd.). The chromatogram was developed by a light petroleum-benzene mixture (1/1, v/v). The alumina column was cut to separate different coloured zones, and each portion was eluted with petroleum ether containing 2–3% (v/v) ethanol, and made up to volume. The pigments in the eluates were determined colorimetrically using 0·04% $K_2Cr_2O_7$ as standard.

For the study of absorption spectra, the separated pigments were purified by two chromatographic adsorptions. The absorption spectra of the purified pigments were studied in light petroleum using a Hilger-Nutting visual spectrophotometer. The pigments constituting the different zones were as follows:

Zone I. This was wide and orange yellow in colour. The pigment was extracted and shown to be identical with cryptoxanthin (maxima in light petroleum, 483 and

Table 1. *Carotenoids of loquat* (Eriobotrya japonica *Lindl.*)

(The figures denoting the pigment content refer to μg./g. of the fresh fruit pulp.)

No.	Variety	Sum of individual pigments (μg./g.)	Xanthophylls other than cryptoxanthin (μg./g.)	(% of total)	Cryptoxanthin (μg./g.)	(% of total)	Neo-β-carotene U (μg./g.)	(% of total)	β-carotene (μg./g.)	(% of total)	Neo-β-carotene B (μg./g.)	(% of total)
1	Pale yellow	5·1	1·4	27·4	1·1	21·5	—	—	2·1	41·2	0·5	9·8
2	Yellow	5·8	1·8	31·0	1·0	17·2	0·3	5·2	2·3	39·6	0·4	6·9
3	Orange yellow	10·5	3·2	30·5	2·4	22·8	—	—	4·1	39·0	0·8	7·6
4	Pale orange	35·7	3·9	10·9	5·6	15·7	1·8	5·0	20·8	58·2	3·6	10·1
5	Orange	37·0	6·6	17·8	12·5	33·8	0·9	2·4	13·5	36·5	3·5	9·4
6	Deep orange	56·5	4·7	8·3	11·6	20·5	1·6	2·8	33·7	59·6	4·9	8·7

450·5 mμ.) since it yielded a single band on mixed chromatography with cryptoxanthin obtained from yellow maize (Sadana, 1946).

Zone II. This was an orange-coloured band. The pigment was identified as neo-β-carotene U (maxima in light petroleum 480 and 450 mμ.; Sadana, 1949).

Zone III. This was reddish orange in colour. The pigment was extracted and identified as β-carotene by its absorption spectrum (maxima in light petroleum 482 and 452 mμ.).

Zone IV. This was a brownish yellow band. The pigment was extracted and identified as neo-β-carotene B (maxima in petroleum 470 and 443 mμ.). It yielded a single band on mixed chromatography with neo-β-carotene B prepared by HCl treatment of β-carotene (Sadana, 1949).

Results of analyses of six varieties of loquats varying in colour from pale yellow to deep orange are shown in Table 1.

SUMMARY

Pigments separated chromatographically and identified from different varieties of loquats include, besides 'xanthophylls other than cryptoxanthin', cryptoxanthin, β-carotene, neo-β-carotene U and neo-β-carotene B. Among the pigments present, cryptoxanthin and β-carotene are the principal ones forming 15·7–33·8 and 36·5–59·6 % of the total pigments.

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Improved Separation of Sugars on the Paper Partition Chromatogram

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Our object has been to develop methods for the quantitative analysis of the mixtures of sugars present in the polysaccharides of the cell wall of fruits; these mixtures frequently include glucose, galactose, xylose, mannose and arabinose, as well as galacturonic and glucuronic acids. The work of Partridge (1946, 1948) has demonstrated the effectiveness of the paper chromatogram as a means of separating reducing sugars, whilst the work of Hirst, Jones & Flood (1947) and Hawthorne (1947) has shown that the amounts of sugars can be estimated within ± 5 % once they have been separated on the paper chromatogram.

It was found in preliminary experiments that none of the solvents previously used in this work was in fact capable of separating all the sugars known to be present in the mixtures under investigation. A large number of solvents was then investigated, and this study led not merely to the development of two new solvents having the required specificity, but also to a more selective method of operating the paper partition chromatogram. The new method involves the use of solvents in which sugars give R_F values of about 0·2. Under such conditions the selectivity defined in terms of the ratio of the R_F values for the pair of sugars to be separated is much improved. Separation of sugars with such low R_F values would need exceedingly long papers if run in the normal manner, but by allowing the solvent to drip off the end of the paper the difficulty has been avoided. Solvents are chosen which have a low viscosity so that the solvent flows quickly through the paper.

To obtain solvents which give R_F values about 0·2 for any particular pair of sugars, it is convenient to use three-component systems so that the partition coefficient, and with it the R_F value, can be adjusted to the desired value. Such solvents have been used previously by Martin & Synge (1941), Isherwood (1946) and Partridge (1946, 1948). Usually two of the components are immiscible whereas the third is miscible with either of the other two. By varying the amount of the third component it is possible to adjust the partition coefficient over a wide range. The two new solvents, ethyl acetate-pyridine-water and ethyl acetate-acetic acid-water are mixtures of three components and in addition have a low viscosity.

EXPERIMENTAL

Apparatus. The apparatus was similar to that described by Partridge (1948). The main difference was that a small hole was bored through the lid of the chamber immediately above one end of the stainless steel trough so that a pipette could be inserted to deliver solvent to the trough. The hole was normally closed with a tight fitting bung. Whatman no. 1 filter paper was used for the strip chromatograms, one end being cut to a point to facilitate even drainage when the solvent front had reached the bottom and was dripping from the paper. The sugar solutions used were roughly 1 % (w/v) with respect to each individual sugar and were

applied to the paper with a micropipette (3–7 μl.) so that the size of the spots could be standardized. The whole apparatus was kept in a constant-temperature room at 20° ± 0·25°, as it was found that changes in temperature altered the R_F values for the sugars in an irregular manner, especially if the critical temperature of the mixture of solvent and water was close to that of the room.

Reagents. The phenol and *s*-collidine were purified as described by Partridge (1946, 1948). The *sec.*-butanol was purified by distillation through a vacuum-jacketed fractionating column, the middle third of the distillate being used for the paper chromatograms. This fraction had a constant boiling point. *iso*Butyric acid was treated similarly. The critical temperature of a mixture of the purified *iso*butyric acid and water was found to be 24·3°. The accepted figure is 24·5°.

Solvents which contained only one component besides water were equilibrated against water at 20°. In the case of solvents containing three components, definite volumes of each component were mixed and when equilibrium between the two phases that resulted had been attained, the non-aqueous phase was separated and used as solvent without further treatment.

Procedure. The spots of sugar solution were added to the paper from a micropipette (3–7 μl.) in a similar manner to that described by Hawthorne (1947). The papers were allowed to dry, and were then hung from the trough in the chamber, no solvent being present. Sufficient of the non-aqueous and aqueous phases was now added to the bottom of the chamber to ensure that the papers would reach equilibrium with the solvent. The lid was placed in position and the apparatus left overnight. After this preliminary equilibration the bung was removed from the small hole in the lid, and solvent added to the trough from a pipette, care being taken to avoid loss of the vapour from inside the chamber. The bung was then replaced. The subsequent treatment of the papers follows closely that described by Partridge (1948).

RESULTS

Variation of R_F value with solvent

Results for rhamnose, xylose and glucose are recorded in Table 1. These sugars were chosen because they were conveniently spaced within the range of R_F values obtained for the simple sugars. The solvent front was not allowed to run off the paper.

These results showed that the absolute R_F values were directly dependent upon the molar fraction of water in the non-aqueous phase. They also showed that, as the molar fraction of water in the non-aqueous phase decreased, the ratio of the R_F values for the pairs of sugars approached a definite maximum. For practical purposes this maximum was reached when the R_F values were less than 0·2, which meant that the possibility of separating two similar sugars increased as the absolute R_F values were reduced to 0·2. A paper chromatogram operated under this restriction did not give a sufficient separation of the sugars if the solvent front remained on the paper because the distance between the centres of the spots was often not more than 1–2 cm. However, if the solvent front was allowed to run off the paper so that the spots moved 10–20 cm. then the distance between the centres increased to 5–10 cm.

Separation of the sugars

From the preceding paragraph it appears that the best conditions for the separation of the sugars are when the R_F values are less than 0·2 and the solvent front is allowed to run off the end of the paper so that the spots move 10–20 cm. The main drawback is that the time required for the spots to move apart on the paper can be considerable if the solvents used have a high viscosity, e.g. *cyclo*hexanol. It is essential, therefore, to use solvents which have a low viscosity in order that the solvent front may move as quickly as possible. A comparison of the viscosity of various solvents with the rate of movement of the solvent front is given in Table 2. The viscosities given are those for pure liquids while the velocity of the solvent front is for the same liquid saturated with water. The influence of viscosity on the separation of the sugars has been calculated by combining the figures for the velocity of the solvent front with the R_F values given in Table 1, the assumption being made that the rate of flow of solvent through the paper and the R_F value remain constant.

Table 1. *Variation of R_F values of sugar with solvent at* 20°

Solvent	Water in non-aqueous phase. Mol. fraction	R_F value			R_F Rhamnose / R_F Xylose	R_F Xylose / R_F Glucose
		Rhamnose	Xylose	Glucose		
Ethyl acetate	0·132	0·01	0·00	0·00	—	—
Methyl acetate	0·269	0·075	0·04	0·00	1·9	—
Methyl ethyl ketone	0·305	0·095	0·05	0·0025	1·9	2·0
Benzyl alcohol	0·387	0·11	0·06	0·03	1·8	2·0
*cyclo*Hexanol	0·432	0·15	0·06	0·03	2·5	2·0
m-Cresol	0·471	0·27	0·15	0·07	1·75	2·1
n-Butanol	0·508	0·22	0·125	0·07	1·7	1·8
tert.-Amyl alcohol	0·609	0·32	0·245	0·145	1·30	1·7
Phenol	0·668	0·59	0·44	0·39	1·34	1·13
Quinoline	0·680	0·41	0·31	0·19	1·32	1·6
sec.-Butanol	0·707	0·41	0·31	0·26	1·32	1·27
*iso*Butyric acid	0·778	0·51	0·44	0·37	1·16	1·19
s-Collidine	0·846	0·59	0·50	0·44	1·18	1·14

Table 2. *Calculated distance the centre of a sugar spot should move in 24 hr. for different solvents at 20°*

Solvent	η (Centipoises)	Velocity of solvent front (cm./hr.)	Distance (cm.) Solvent front	Rhamnose	Xylose	Glucose
Methyl acetate	0·38	12	288	21·6	11·5	0·0
Ethyl acetate	0·45	10	240	2·4	0·0	0·0
Methyl ethyl ketone	0·43	10·8	258	25·5	12·9	6·4
Water	1·0	3·75	—	—	—	—
*iso*Butyric acid	1·3	2·25	54	27·5	23·7	19·9
n-Butanol	3·0	2·6	62	13·6	7·8	4·4
sec.-Butanol	3·7	2·6	62	25·6	19·2	16·2
tert.-Amyl alcohol	4·7	1·25	30	9·6	7·3	4·3
Benzyl alcohol	5·8	1·6	38·4	4·2	2·3	1·1
Phenol	11·7	1·5	36	21·2	15·8	14·1
m-Cresol	20·0	1·1	26·5	7·1	4·0	1·8
*cyclo*Hexanol	68·0	0·65	15·6	2·3	0·9	0·4

Table 3. *Separation of sugars using three-component solvents*

Mixture prepared (in vols.) Non-aqueous phase used as solvent	Duration of test (hr.)	Movement of the centre of the sugar spot from the starting point (cm.) Rhamnose	Xylose	Arabinose	Mannose	Fructose	Glucose	Galactose	Galacturonic acid
tert.-Amyl alcohol 3, Water 3, Acetic acid 1	20	8·6	7·0	5·4	5·9	5·9	4·6	4·1	3·2
Benzyl alcohol 3, Water 3, Acetic acid 1	22	12·8	7·8	7·5	5·7	7·2	4·7	4·6	2·6
Chloroform 4, *Water 4, Methanol 7	24	28·6	23·3	20·5	18·6	19·3	16·3	15·9	6·6
Ethyl acetate 16, *Water 16, Methanol 9	14	6·5	5·4	4·7	3·8	4·0	3·2	3·1	1·1
Ethyl acetate 2, Water 2, Pyridine 1	22†	37·9 (0·49)	33·6 (0·38)	29·2 (0·33)	28·9 (0·32)	28·4 (0·32)	26·0 (0·28)	20·9 (0·235)	6·7 (0·025)
Ethyl acetate 3, Water 3, Acetic acid 1	39†	28 (0·34)	25·3 (0·265)	20·5 (0·22)	17·6 (0·195)	— —	14·3 (0·17)	12·4 (0·14)	12·0 (0·13)
Ethyl acetate 2, *Water 2, Dioxan 3	14	30·1	26·2	22·4	22·4	21·3	20·8	17·7	5·1

* These mixtures only just formed two phases, and as the presence of the sugar markedly increased the mutual solubility of the two phases, the spots 'tailed' badly. The separation between the sugar spots was, therefore, less than the figures suggest.

† R_F values are given in parentheses.

In practice the separations achieved are much less than is shown in Table 2, the main reason being that when the solvent commences to drip from the end of the paper, the rate of flow of solvent is reduced to about half the initial value. The relative separations, however, remain substantially the same, though they do not entirely correspond to the R_F values measured when the solvent front is still on the paper. The explanation probably lies in the fact that the distribution of the solvent throughout the paper strip is modified if liquid accumulates at the bottom (cf. Consden, Gordon & Martin, 1944).

None of the solvents listed in Table 2 is ideal. One or two give excellent separations of the pentoses, but do not give an appreciable separation of the hexoses in a convenient time (24 hr.). The addition of a third component which is soluble in both phases will tend to increase the solubility of hexoses in the non-aqueous solvent and thereby increase their R_F values. The separation of the pentoses will be relatively less affected. The results obtained with a number of three-component mixtures are given in Table 3.

Of these mixtures ethyl acetate-pyridine-water and ethyl acetate-acetic acid-water were selected as they fulfilled the requirements for which this work was undertaken.

Analysis of mixtures of sugars

In a mixture containing arabinose, xylose, glucose, galactose, galacturonic acid and mannose, it was possible to detect all the components with certainty using one or other of the above solvent mixtures. Of these sugars arabinose could not be distinguished from mannose in ethyl acetate-pyridine-water, and galactose and galacturonic acid could not be separated in ethyl acetate-acetic acid-water. If a pair of sugars had very different R_F values separation was completed very rapidly. Thus with ethyl acetate-pyridine-water a mixture of glucose and xylose was completely separated in 2 hr. If only a small proportion of one sugar was present it was advantageous to work with a concentrated solution though the more concentrated the solution used the larger the spot after development. The use of concentrated solutions was possible with the solvents described above because the distance between the centres of the sugar spots was 3–5 cm. Thus, using ethyl acetate-acetic acid-water as solvent, mannose was identified in the presence of 10 times its concentration of glucose and 100 times its concentration of xylose. The total concentration of sugars in the solution used for making the spot was 20 % (w/v), and the diameter of the xylose spot after the chromatogram was developed was 4–5 cm.

Quantitative analysis of the sugars on the paper chromatogram after they had been separated using either of these solvents was carried out successfully either by the method described by Hirst *et al.* (1947) or by the method described by Hawthorne (1947). We preferred, however, a combination of the two methods. A known amount of the sugar solution was added to the paper from a micropipette (Hawthorne, 1947), and then the sugars separated and isolated following the method described by Hirst *et al.* (1947). Analysis of four mixtures by this method is given in Table 4.

Table 4. *Analysis of mixtures of sugars*

	Amount added (μg.)	Amount recovered (μg.)				
		Individual experiments				Mean
Mixture 1						
Xylose	68·5	61·6	70·4	74·0	67·2	67·0
Arabinose	68·5	77·4	71·3	67·8	67·9	71·1
Mannose	68·5	69·4	76·4	67·3	65·5	69·4
Glucose	68·5	67·9	59·1	77·6	65·3	67·5
Galactose	68·5	74·4	68·9	66·6	71·8	70·4
Mixture 2						
Xylose	68·5	71·0	66·4	75·0	68·1	70·1
Arabinose	68·5	68·0	69·4	74·1	70·4	70·4
Glucose	68·5	75·4	71·1	66·8	64·1	69·3
Galactose	68·5	74·6	61·9	77·1	65·2	69·8
Galacturonic acid	68·5	63·2	85·8	74·2	73·6	74·2
Mixture 3						
Xylose	212	222	215	217	211	216
Arabinose	24·0	25·5	29·2	30·1	20·1	26·4
Glucose	439	433	428	421	426	427
Galacturonic acid	44·3	39·4	46·1	41·8	44·7	43·0
Mixture 4						
Xylose	19·0	19·8	18·4	21·0	23·4	20·7
Arabinose	414	415	391	421	400	406
Glucose	39·8	33·9	45·3	42·1	38·4	39·9
Galacturonic acid	204	208	200	210	214	208

Mixture 1 was separated using ethyl acetate-acetic acid-water as solvent. The time required for the separation of the sugars was 48 hr. Mixtures 2, 3, 4 were separated using ethyl acetate-pyridine-water as solvent. In the case of mixtures 3 and 4 the composition of the solvent was slightly modified, the components being in the ratio of 5, 2, 5 instead of the ratio of 2, 1, 2 respectively as described in Table 3. The modified solvent gave slightly better separations than the original. The papers were dried at room temperature overnight because it was found that drying at the usual temperature of 60–100° in the presence of pyridine appeared to destroy a large part of the sugar present.

Mixtures 3 and 4 are examples of the separations achieved when some of the sugars are present only in small proportions.

DISCUSSION

If we consider the separation of the sugars after they have moved a definite distance down the paper chromatogram we find that the best separations are obtained with solvents which give R_F values less

than 0·2, the solvent being allowed to run off the end of the paper. This observation is in agreement with predictions which can be made by applying to the paper chromatogram the equation first deduced by Martin & Synge (1941) for the relation between the complete separation of two solutes (99·8 % of one and 0·2 % of the other) and the characteristics of the partition column:

$$\frac{A_L+\alpha A_S}{A_L+\beta A_S}=\frac{r-3\sqrt{r}+2\cdot25}{r+3\sqrt{r}+2\cdot25},$$

where α and β are the partition coefficients of the two solutes, A_L is the area of cross-section of the mobile phase, A_S is the area of cross-section of the non-mobile phase and r represents the number of 'theoretical plates' the first solute has passed. At any particular place in the column $\frac{r-3\sqrt{r}+2\cdot25}{r+3\sqrt{r}+2\cdot25}$ is a constant and represents the maximum value that $(A_L+\alpha A_S)/(A_L+\beta A_S)$ can have if the two solutes are to be just separated. If A_L is small in relation to αA_S or βA_S the expression reduces to α/β. This is true when the partition coefficients are large. Now if we assume that the ratio α/β is likely to be sensibly constant for solvents of a similar type it follows that $(A_L+\alpha A_S)/(A_L+\beta A_S)$ reaches its minimum value when the partition coefficients are large, and that if two solutes are only just separated when the partition coefficients are large they will not be separated when the partition coefficients are small. The assumption that the ratio α/β is sensibly constant for solvents of a similar type is strongly suggested by analogy with the data given by Cohn & Edsall (1943) on the solubility of amino-acids and their derivatives in various organic solvents and in water. It is important, therefore, to choose a solvent for which the partition coefficients are large so that the maximum separation is achieved. In paper chromatograms A_L/A_S is about 4 (Consden *et al.* 1944) so that α and β must be at least 10–15. This corresponds to an R_F value of 0·2–0·3.

In practice it is not always easy to find a two-component solvent (water is always one component) which will give R_F values of about 0·2 for a particular pair of sugars. Three-component solvents are, however, much more flexible in this respect. Originally these solvents were introduced in order to obtain a more convenient spread of R_F values for pentoses and hexoses than was possible with two-component solvents, but they can be readily applied to the separation of sugars with similar R_F values. The R_F value of a sugar can be altered by changing the amount of water dissolved in the organic phase; the general relationship between the amount of water in the organic phase and the R_F value is clearly shown by the figures in Table 1. The addition to a two-component system of a third substance, which is readily soluble in both phases, increases the amount of water held in the non-aqueous phase, whereas addition of a substance which is soluble only in one phase diminishes the amount of water. Thus, by using the organic phase from a mixture consisting of two organic solvents and water, one of the organic solvents being miscible with water and the other immiscible, it is possible to alter the R_F value within wide limits. The R_F value for any particular pair of sugars can, therefore, be adjusted to about 0·2.

The apparatus used in the present experiments has the advantage that during the development of the chromatogram the phases on the paper are not disturbed. In the method described by Partridge (1948) the lid of the chamber is removed when the trough is filled with liquid, and the atmosphere is, therefore, initially unsaturated, and requires several hours at least to reach equilibrium with the solvent. During this time the advancing solvent front is losing solvent to the atmosphere, and particularly in the case of three-component systems may not have the same composition as the solvent in the trough. In extreme cases with certain mixtures the two phases become miscible and then all the sugars travel down the paper just behind the solvent front. By filling the trough without opening the chamber after the paper and atmosphere have reached equilibrium with the solvent, we can avoid any disturbance of the phases on the paper.

SUMMARY

1. A number of solvents have been examined for the separation of complex sugar mixtures on the paper chromatogram. It has been shown that two three-component mixtures, ethyl acetate-acetic acid-water and ethyl acetate-pyridine-water are more satisfactory than any previously described solvents for the qualitative and quantitative determination of the sugars in mixtures of the simple hexoses and pentoses.

2. Some of the factors which influence the separation of the sugars on the paper chromatogram are described and analyzed. It is shown that improved separations can be obtained by using solvents which give low R_F values, the solvent front being allowed to run off the paper. The use of a solvent with a low viscosity shortens the time needed for analysis because the solvent flows more rapidly through the paper.

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Zinc Uptake by *Neurospora*

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Among the minor constituents of living tissues zinc is one of great interest (see Lehman, 1939; Broh-Kahn & Mirsky, 1948; Warburg, 1948; Bertrand, 1948). It constitutes 0·33 % of carbonic anhydrase (Keilin & Mann, 1940), and it is a constituent of uricase which contains 1·3 % of zinc (Davidson, 1938; Holmberg, 1939) and possibly of other enzymes. It was found to be essential for growth and citric acid formation of *Aspergillus niger* (Bernhauer, 1928), for growth and fumaric acid production of *Rhizopus nigricans* (Foster & Waksman, 1939) and for growth and subtilin formation by *Bacillus subtilis* (Feeney, Lightbody & Garibaldi, 1947).

The presence of 0·2 mg. of zinc in the food of mice is stated by Bertrand (1948) to prolong the life of the animals from 2–3 weeks to 2–2·5 months.

Wassiljew (1935) demonstrated that the effect of zinc was not the same for biochemically different strains of *A. niger*, and he went so far as to suggest a characterization of *A. niger* strains based on their reactivity to zinc. Foster & Waksman (1939) interpret the function of zinc as catalyzing a more complete destruction of the glucose molecule with a consequent greater efficiency of energy utilization by the fungus.

In connexion with studies on the growth of *Neurospora crassa* we determined the amount of zinc necessary to obtain a maximum growth of that mould.

METHODS

Throughout the experiments mycelium from one spore was used. This was produced by crossing the two wild-type strains, nos. 25*a* and 1*A* of Beadle (1945). The culture solution was that described by Ryan, Beadle & Tatum (1943); it was autoclaved, and the cultures were treated under sterile conditions. Inocula were taken from cultures 4–5 days old.

Labelled zinc as chloride and varying amounts of non-radioactive zinc as sulphate were added to culture solutions at the start of the experiments. From the specific activities of the zinc present in the culture solution and in the mould, and the amount of zinc added to the culture solution, the zinc content of *Neurospora crassa* was calculated. We are much indebted to the Atomic Energy Commission of the U.S.A. and the Chief of the Isotope Branch, Dr Poul Aebersold, for supplying the zinc containing the radioactive isotope ^{65}Zn.

The activity of the ^{65}Zn taken up by *Neurospora* was less than 1 μcurie, and thus the radiation emitted could hardly produce any noxious action on the mould.

After centrifuging and thorough washing the *Neurospora* was dried at 105°, brought into solution by wet ashing, 5 mg. zinc as sulphate added as carrier, and the zinc precipitated with 8-hydroxyquinoline. The precipitate was filtered through a perforated aluminium dish covered by a layer of filter paper, and the dish placed under a Geiger-Müller counter of the type described by Madsen (1945). A sample of the active $ZnCl_2$ solution was treated in the same way. By comparing the activities of this 'standard' precipitate of known zinc content and that of the above-mentioned precipitates, the percentage of the zinc originally present in the culture solution which accumulated in the mould was calculated.

RESULTS

The results of some of our experiments, in which each *Neurospora* was grown in 150 ml. culture solution of varying zinc content for 5 days at 25°, are given in Table 1. This table and Fig. 1 show that the presence of 1 part of zinc in about 30,000 parts of dry mould is sufficient for an almost maximal growth, and the presence of 12·5 μg. zinc in 150 ml. culture solution suffices to give that zinc content. It is advisable to add a somewhat larger amount of zinc to the culture solution, e.g. 200 μg./l. as recommended by Ryan *et al.* (1943) or 450 μg./l. as stated by Mitchell & Houlakan (1946).

Table 1. *Amount of zinc taken up by* Neurospora

Zinc in culture solution (μg.)	Wt. of mould (mg.)	Zinc (μg./mg. dry mould)	Percentage of the ^{65}Zn content of culture solution present in 1 mg. dry mould
2·5	106·6	0·021	0·84
	106·9	0·013	0·50
	97·8	0·012	0·48
	90·0	0·014	0·55
Average	**100·3**	**0·015**	**0·59**
12·5	175·2	0·033	0·26
	169·7	0·033	0·26
	151·7	0·035	0·28
	144·3	0·040	0·32
Average	**158·9**	**0·035**	**0·28**
50·0	208·6	0·090	0·18
	178·8	0·11	0·21
	168·1	0·10	0·19
	166·7	0·11	0·22
Average	**180·5**	**0·10**	**0·20**
250·0	153·8	0·13	0·033
	172·3	0·12	0·043
	147·7	0·11	0·044
	161·9	0·14	0·055
Average	**158·9**	**0·13**	**0·049**
2000·0	186·3	0·30	0·015
	184·5	0·34	0·017
	158·3	0·38	0·019
	164·0	0·38	0·019
Average	**168·3**	**0·35**	**0·018**

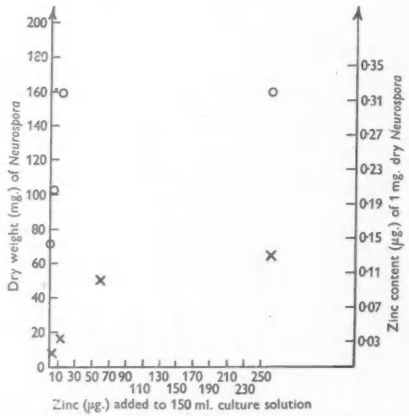

Fig. 1. Effect of varying zinc content of the culture solution on the dry weight (○) and zinc content (×) of *Neurospora* after the lapse of 5 days.

Fig. 1 shows the very pronounced increase in weight of the mould, from 71 to 100 and 159 mg., after the addition of 2·5 and 12·5 mg. of zinc respectively to the culture solution. In experiments of shorter duration the effect of zinc on the increase in weight was still more pronounced (Fig. 2). After 48 hr. 28 mg. were obtained in the absence of added zinc, while adding 30 μg. of zinc to a 150 ml. culture produced 83 mg. of mould.

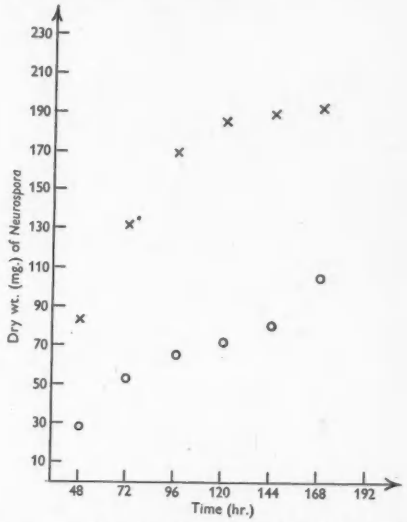

Fig. 2. Dry weight of *Neurospora* without (○) and after (×) addition of 30 μg. of zinc.

After 5 hr., a 150 ml. culture solution containing 2·5 μg. zinc produced 100·3 mg. and one with 12·5 μg. zinc, an almost maximal weight of 158·9 mg. In the former case 1 mg. *Neurospora* contained 0·015 μg. zinc; in the latter roughly twice that amount (0·035 μg.). An increase in the zinc content of the culture solution to 2000 μg. did not increase the weight of *Neurospora* observed after the lapse of 5 days (Table 1). The capacity of the fungus to take up zinc was, however, not exhausted, as shown by the fact that an eightfold increase in the zinc concentration of the solution resulted in an almost threefold zinc uptake by the mould.

Feeney *et al.* (1947) determined the amount of zinc to be added to the culture solution in order to obtain maximal growth and maximal antibiotic formation in cultures of *Bacillus subtilis*. This was found to be 0·40 mg./l., while in the case of *Neurospora crassa* an almost maximal growth was observed by us in the presence of as little as 0·08 mg./l. of zinc in the culture solution.

Migration of zinc from the Neurospora *into the culture solution*

By making use of isotopic indicators the migration of ions from the plant into the culture solution has been observed in several cases, and the effect on the

migration process of unlabelled ions in the solution has also been studied. Minute amounts of labelled lead, when taken up by the roots of *Vicia Faba*, could to a large extent be removed by an excess of non-labelled lead added to the nutrient solution, but to a minor extent only if the solution did not contain lead (Hevesy, 1923).

Mullins & Brooks (1939) placed cells of *Nitella coronata* first in a solution containing radioactive potassium (^{42}K) and later in solutions of different chlorides. Sodium and lithium were found to be much less effective in removing labelled potassium than potassium itself. We investigated the exodus of labelled zinc into the culture solution both in the absence and presence of zinc in the solution in which the mould containing labelled zinc was placed.

In these experiments *Neurospora* grown for 5 days at 28°, after repeated washing with distilled water, was divided into five approximately equal parts. One part was kept as a control, two parts immersed separately in 150 ml. culture solution containing no zinc for 24 hr. at 12°, while two parts were kept under similar conditions in a culture solution containing 30 μg. zinc.

Table 2. *Migration of labelled zinc from* Neurospora *into the culture solution*

Sample	Activity
Control	100
Neurospora after being kept in zinc-free solution for 24 hr. at 12°	88, 84
Neurospora after being kept in zinc-containing solution for 24 hr. at 12°	74, 75
Zinc-free solution after 24 hr.	9, 10
Zinc-containing solution after 24 hr.	14, 19
Penultimate wash water	1

In all our experiments more labelled zinc left the mould when placed in a solution containing zinc. Table 2 shows the results of a typical experiment.

Uptake of cobalt by Neurospora

We compared the uptake of Co^{++} (using ^{58}Co as an indicator) with that of zinc by *Neurospora*. Four moulds were grown for 5 days at 28° in the presence of 25 μg. of cobalt in the culture solution. They were found to take up 0·013, 0·011, 0·011 and 0·011 % of the cobalt content of the solution per mg. of their dry weight (i.e. 0·0033, 0·0028, 0·0028 and 0·0028 μg.). The cobalt uptake corresponds to only about one-twentieth of the uptake of zinc under similar conditions, showing that the moulds do not possess such an effective mechanism for fixing cobalt as they do for zinc.

SUMMARY

1. By making use of labelled zinc (^{65}Zn) the amount of zinc necessary to obtain maximal growth of *Neurospora crassa* was studied.

2. Maximal growth requires that at least 1/30,000 part of the dry weight of the *Neurospora* be composed of zinc, and can be obtained by raising the mould in a culture solution containing 0·08 mg. or more of zinc/l.

3. When placed in a culture solution containing inactive zinc more labelled zinc migrates from *Neurospora* into the solution at 12° than in the absence of zinc.

4. Cobalt uptake, studied by making use of labelled cobalt (^{58}Co), amounts only to one-twentieth of the uptake of zinc under corresponding conditions.

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Accumulation of Glutamic Acid in Isolated Brain Tissue

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Van Slyke (1913) was the first to show that most animal tissues have a far higher concentration of free amino-acids than has blood plasma. For example, 75, 73 and 64 mg. non-protein amino N were found in 100 g. of dog liver, kidney and spleen respectively, and only about 4–5 mg. in 100 g. of blood plasma (Van Slyke & Meyer, 1913). Hamilton (1945) and Krebs, Eggleston & Hems (1949) have reported comparable differences for the distribution of glutamine and glutamic acid between tissues and plasma. Since amino-acids can travel readily from tissue to plasma, and plasma to tissue, special mechanisms must exist which control the rate of transport and maintain high concentration gradients.

This paper is concerned with a study of this mechanism. When slices of brain and other tissues are kept in a saline medium containing glutamate the concentration of this amino-acid in the tissue rises under some conditions and falls under others. Transport of glutamate can occur against a considerable concentration gradient. The changes observed are sufficiently large to allow accurate measurements, and the factors which control the concentration gradient between tissue and medium can thus be investigated under relatively simple conditions.

EXPERIMENTAL

Handling of tissue. In the present experiments it was necessary to measure the fresh weight of the tissue before incubation in a nutrient medium. It was therefore essential to avoid contact of the tissue with medium during the slicing operations. After preliminary experiments the following procedure was adopted. The brain of a guinea pig was removed from the animal immediately after death and placed on dry filter paper for slicing. Slices of the grey matter were made with a dry cutting blade by the method of Deutsch (1936), or of Stadie & Riggs (1944), and were weighed on a torsion balance. Samples of *c.* 0·15 g. fresh weight were placed in the medium in the main compartment of each conical Warburg cup, in which they were shaken at 40° for specified periods. At the end of the incubation the cups were placed in ice water to stop metabolic activity and the slices were collected as quickly as possible. After draining off the medium along the wall of the manometer vessel the slices were again weighed on the torsion balance. They were then ground in a mortar with 2·5 ml. of 0·4 N-HCl. The remaining medium was acidified with 0·25 vol. of 2 N-HCl immediately after the removal of the tissue and ice-cooled. The sum of L-glutamic acid and L-glutamine was determined in a measured fraction of this solution and in 2 ml. of the suspension of ground tissue. In each experiment a sample of slices which had not been incubated was treated and analyzed in the same way as the incubated material.

Rabbit brain was found to be less suitable because slices of this material broke up into many particles when shaken in saline media, whilst guinea-pig brain slices remained coherent. Smaller animals, such as rats or pigeons, did not yield enough slices for a series of experiments on one brain. One guinea-pig brain was usually sufficient for five samples of 0·15 g. each.

All data on tissue weights given in this paper refer to fresh weights.

Medium. Phosphate saline without Ca (Krebs & Eggleston, 1940) was used as the standard medium. Substrates were added in the form of neutral 0·2 M-solutions and the total volume of the medium, including the added substrates, was 4 ml. The substrates were added to the medium from the side arm immediately before the manometers were attached to the water bath. Unless otherwise stated, the gas space of the vessels was O_2.

Analytical methods. The decarboxylase method of Gale (1945), as modified by Krebs (1948), was used for the determination of the sum of L-glutamic acid and L-glutamine. It is specific for these two substances. D-Glutamate is not attacked by the bacterial decarboxylase. As a rule no effort was made to differentiate between the two substances and in the following tables 'glutamic acid' or 'glutamate' refer to the sum of L-glutamic acid and L-glutamine except where it is specifically stated that a differential determination was carried out.

Calculation. A difficulty in the quantitative interpretation of the measurements arose from the finding that the thoroughly drained brain slices collected at the end of the incubation always weighed more than the fresh slices as placed in the medium. The increase was usually about 25–50% under aerobic, and 50–75% under anaerobic, conditions, and could have been due either to the adherence of saline medium to the tissue or to swelling of the latter. Elliott (1946) has in fact shown that brain tissue swells when immersed in saline media and this is confirmed by observations reported in this paper; however, the weight increase observed under our conditions was probably due to both swelling and wetting.

If the concentration of glutamate in the slices is calculated on the assumption that the weight increase is due to adherent medium, higher values are obtained than if it is assumed to be due to swelling. Examples showing the extent of the differences obtained by the two methods of calculation are given in Tables 1 and 2. In contrast, the figure for

the total amount of glutamate taken up by the slices is not much affected by the method of calculation as long as the concentration of glutamate in the medium is much lower than in the slices, which was the case in most experiments. To what extent the calculated results vary with the concentration of glutamate in the medium can be seen from the data given in columns 3 and 5 of Table 2.

In all cases, except in the specified columns of Tables 1 and 2, the amount of glutamic acid in the slices was calculated on the assumption that all the weight increase was due to adherent medium. The concentration of glutamic acid in the medium at the end of the incubation period was determined in every experiment so that the correction for the adherent medium could be made. Any errors arising from the incorrectness of the assumption would cause the uptake of glutamate by the tissue to appear too small, and the concentration of glutamate in the slices to appear too high.

The amount of glutamic acid removed from the medium was in most experiments larger than the amount which accumulated in the tissue. This is taken as an indication that the tissue had converted some glutamic acid into other substances. This fraction of glutamic acid (glutamic acid removed from medium minus glutamic acid accumulated in tissue) will be referred to as 'glutamic acid metabolized'; in accordance with general usage, the removal of glutamic acid from the system will be denoted by the minus sign. In some cases, indicated by the positive sign, glutamic acid in the system 'medium plus slices' increased. Where the determination of 'glutamic acid metabolized' rests on the measurement of a difference, in the presence of a total amount of *c.* 400–900 μl. glutamate, small values for glutamic acid metabolized (below 20 μl.) are of doubtful significance (1 μmol. glutamic acid $\equiv$ 22·4 μl.; cf. Krebs, 1948).

RESULTS

Accumulation of L-*glutamic acid in slices of brain cortex.* On incubation with glutamate, in the presence of glucose, the amount of glutamate in the slices increased progressively during the first 60 min. up to about two and a half times the initial value (Table 1, cols. 4 and 5). At the same time glutamate disappeared from the medium (Table 1, cols. 8 and 9). The differences in the figures given in columns 5 and 9 show that, except for the first 20 min. period, where the measurement was not very accurate owing to the relatively small change, considerable amounts of glutamate were metabolized under the conditions of the experiment.

The transport of glutamate into the tissue occurred against a concentration gradient (see Table 1, cols. 10–12). The computations of this gradient give somewhat, but not decisively, different figures according to the assumptions on which they rest (cf. Table 1, cols. 10 and 11).

During the 60–80 min. period the glutamic acid concentration dropped in the slices whilst it increased in the medium. Thus the mechanism of transporting glutamic acid into the tissue or of maintaining the concentration gradient had lost its efficiency after 60 min., presumably owing to tissue damage. In later experiments on brain tissue where rates were to be measured, the incubation period was usually 40 min.

A comparison of the rate of accumulation of glutamate in brain tissue with the metabolic processes shows that the average rate of accumulation in the slice was 3·7 μl. glutamic acid/mg. dry wt./hr. for the first 20 min. and 3·1 for the first 60 min. The average rate of removal from the solution, on the basis of the 60 min. value, was 4·9. Thus the rate of glutamate removal by metabolic reactions was 1·8, or about one third of the rate of removal from the solution. For the purpose of these calculations it was assumed that the dry weight of the non-incubated slices was 20 % of the wet weight.

Effect of the concentration of L-*glutamate.* Increasing the concentration of glutamate in the medium caused an increased uptake by the slices (Table 2, cols. 4 and 6). It is remarkable that at the end of the incubation period the differences between the concentrations in the slices and in the medium were about the same in spite of great differences at the start (see Table 3). The amounts of glutamate

Table 1. *Accumulation of* L-*glutamate in slices of guinea-pig brain cortex*

(0·02 M-Glucose; 0·005 M-L-glutamate. The values given in column 10 were calculated by dividing the corresponding figures in col. 4 by those in col. 2, the values in col. 11 by dividing the figures in col. 6 by those in col. 1.)

Period of incubation (min.)	Wt. of slices/cup: Initial (g.)	Wt. of slices/cup: After incubation (g.)	Wt. increase of slices (g.)	L-Glutamate found in drained slices: Total (μl.)	L-Glutamate found in drained slices: Change (μl.)	L-Glutamate in slices corrected for adherent medium: Total (μl.)	L-Glutamate in slices corrected for adherent medium: Change (μl.)	L-Glutamate in medium: Total found (μl.)	L-Glutamate in medium: Change (μl.)	Concentration of L-glutamate: In slices: As weighed (μl./g.)	Concentration of L-glutamate: In slices: Corrected for adherent medium (μl./g.)	Concentration of L-glutamate: In medium (μl./ml.)
Column ...	1	2	3	4	5	6	7	8	9	10	11	12
0	0·146	—	—	60	—	60	—	428	—	412	412	107
20	0·145	0·210	+0·065	96	+36	91	+31	398	− 30	458	628	100
40	0·143	0·217	+0·074	117	+57	112	+52	327	−101	540	781	82
60	0·148	0·226	+0·078	151	+91	146	+86	282	−146	666	986	71
80	0·149	0·219	+0·070	122	+62	117	+57	292	−136	555	785	73

Table 2. *Effect of* L-*glutamate concentration on the accumulation in slices of brain cortex*

(Period of incubation 40 min.; 0·02 M-glucose; initial amount of L-glutamate in 150 mg. slices, 49 μl.)

Initial amount of L-glutamate in 4 ml. (μl.)	Wt. of slices/cup		L-Glutamate found in drained slices		L-Glutamate in slices corrected for adherent medium		L-Glutamate found in 4 ml. medium after incubation		Final concentration of L-glutamate		
									In slices		
	Initial (g.)	After incubation (g.)	Total (μl.)	Change (μl.)	Total (μl.)	Change (μl.)	Total (μl.)	Change (μl.)	As weighed (μl./g.)	Corrected for adherent medium (μl./g.)	In medium (μl./ml.)
Column ...	1	2	3	4	5	6	7	8	9	10	11
222	0·149	0·224	85	+ 36	84	+35	116	−106	382	561	29
444	0·149	0·221	92	+ 43	86	+37	361	− 83	414	580	90
888	0·144	0·206	114	+ 67	105	+58	733	−155	552	726	183
1776	0·147	0·218	157	+109	134	+86	1607	−169	720	912	402

metabolized (calculated from columns 8 and 4 or 6 of Table 2) did not vary much with the glutamate concentration in the medium. This is not unexpected as the metabolic reactions should depend on the substrate concentration in the tissue, which showed relatively little variation, rather than on the substrate concentration in the medium.

Table 3. *Difference in the concentration of* L-*glutamate between slices and medium*

(Calculated from data given in Table 2. For the purpose of the calculation it is assumed that 1 g. slice equals 1 ml. slice. 'Difference' is concentration in slices *minus* concentration in medium. The minus sign indicates that the concentration in the slices was lower than the concentration in the medium.)

Initial difference (μl./ml.)	Difference after incubation	
	Calculated from cols. 9 and 11 (μl./ml.)	Calculated from cols. 10 and 11 (μl./ml.)
282	353	532
216	324	490
115	368	540
−111	318	510

Effect of various substrates on the fate of L-*glutamate.* When L-glutamate was the only substrate added to brain slices the original concentration of glutamate in the tissue was not maintained but fell (Table 4, Exp. 1). Succinate, fumarate, citrate, α-glycerophosphate, phosphoglycerate or adenosine triphosphate were unable to prevent this fall. In the presence of glucose, fructose, pyruvate, lactate and fructose-1:6-diphosphate the concentration of glutamate rose on incubation. The effect of glucose was greater than that of the other substances, except in one case (Exp. 1) where the effect of glucose was unusually low and where lactate was more effective than glucose.

A comparison of the effects of the above substances with their oxidizability in brain tissue shows no simple correlation. Substances which cause an accumulation of glutamate in the tissue are all oxidizable, but oxidizability alone is not sufficient to support accumulation; glutamate, succinate and α-glycerophosphate, although readily oxidized, were ineffective even in preventing the fall of glutamate in the tissue. Whilst the oxygen uptakes in the presence of glucose, lactate and pyruvate were of the same order, glucose was definitely more efficient in supporting accumulation.

The amount of glutamate metabolized was by far the greatest in the presence of oxaloacetate, transamination being mainly responsible for the removal in this special case (see Exp. 3). The relatively large amount of glutamate metabolized in the presence of fumarate was probably also due to transamination with the oxaloacetate arising by oxidation of the fumarate. In general, substrates which were readily oxidized, like glucose, lactate or pyruvate, reduced the amount of glutamate metabolized, whilst substances which are not readily oxidized in brain tissue had no appreciable effect.

The rate of glutamate removal in the presence of oxaloacetate, in terms of the usual Q values (μl./mg. dry wt./hr.), was 28, and if the reaction had occurred within the brain cells this figure would represent the minimum rate of entry of glutamate into the tissue. On the other hand, the data in Table 1 indicate an actual rate of entry under similar conditions of no more than 5. To clear up this apparent discrepancy experiments were carried out to test whether the transaminase diffuses into the medium and allows the reaction to take place outside the slice. Brain slices (138 mg.) were shaken in glucose saline (4 ml.) for 40 min. in an atmosphere of O_2. The slices were then removed; the medium was centrifuged and 3 ml. of the supernatant were incubated at 40° with 0·2 ml. of 0·02 M-glutamate, 0·4 ml. of 0·02 M-oxaloacetate and 0·4 ml. of saline. After 40 min. the reaction was stopped by the addition of 2 N-HCl (1 ml.) and glutamate was determined in the solution. Of the 890 μl. glutamate added only 244 were recovered. It

Table 4. *Effect of various substrates on the fate of* L-*glutamate in guinea-pig brain cortex*

(Incubation 40 min. Concentration of glutamate 0·01 M, of other substrates 0·02 M unless otherwise stated.)

Exp. no.	Substrates added in addition to glutamate	Initial wet wt. of slices (g.)	Amount of glutamate found in slices: Initially (μl.)	After incubation: Total (μl.)	After incubation: Change (μl.)	Amount of glutamate removed from medium (μl.)	Amount of glutamate metabolized (μl.)
1	None	0·162	59	52	− 7	− 54	− 61
	Glucose	0·165	60	100	+40	− 86	− 46
	Pyruvate	0·160	58	91	+33	− 63	− 30
	L-Lactate	0·168	61	112	+51	− 68	− 17
2	Glucose	0·144	52	148	+96	−208	−112
	Fructose	0·185	67	124	+57	−125	− 68
	Pyruvate	0·167	60	103	+43	−127	− 84
	L-Lactate	0·157	57	101	+44	− 93	− 49
3	Glucose	0·157	55	127	+72	−144	− 72
	Oxaloacetate	0·151	53	57	+ 4	−566	−562
	α-Glycerophosphate	0·194	67	50	−17	− 73	− 90
	Adenosinetriphosphate (0·002 M)	0·162	56	42	−14	− 90	−104
4	Glucose	0·167	65	160	+95	−126	− 31
	Succinate	0·161	63	51	−12	− 73	− 85
	Fumarate	0·169	66	56	−10	−130	−141
	Citrate	0·169	66	42	−24	− 85	−109
5	Glucose	0·153	53	120	+67	−155	− 98
	DL-Phosphoglycerate	0·143	49	42	− 7	− 25	− 32
	Adenosinetriphosphate (0·002 M); DL-phosphoglycerate	0·159	55	57	+ 2	− 59	− 57
	Fructose-1:6-diphosphate	0·197	68	95	+27	− 66	− 39

follows that enough transaminase can diffuse out of the tissue slices to account for the change in glutamate concentration observed in Exp. 3 of Table 4. Thus whilst the slices can retain and even concentrate glutamate they lose the very much larger molecule of the transaminase whose molecular weight has been estimated at 60,000 (Green, Leloir & Nocito, 1945). Further quantitative assays of the glutamic-aspartic transaminase showed that after 40 min. incubation in glucose saline 25% of the enzyme had passed from brain slices into the medium. Whipple & Madden (1944) have adduced evidence which suggests that other large molecules—the plasma proteins—flow readily to and fro between tissue and plasma.

Comparison of L-*glutamate and* L-*glutamine.* After a short period of incubation (15 min.) the amount of glutamate plus glutamine found in the slices was greater when glutamine was the substrate, whilst after 30 min. the concentrations reached with glutamate and glutamine were about equal (Table 5). The data suggest that glutamine is taken up a little more rapidly than is glutamate. In a similar experiment a differential determination of glutamate and glutamine was carried out after an incubation period of 60 min. The method recently described was used

Table 5. *Comparison of the rates of accumulation of* L-*glutamate and* L-*glutamine in guinea-pig brain cortex*

(Glucose 0·02 M.)

Exp. no.	Period of incubation (min.)	Initial wt. of slices (g.)	Substrates added (0·01 M)	Sum of glutamate and glutamine found in slices: Total (μl.)	Sum of glutamate and glutamine found in slices: Increase (μl.)
1	0	0·161	—	56	—
	15	0·166	Glutamate	75	19
	15	0·166	Glutamine	97	41
	30	0·165	Glutamate	96	40
	30	0·167	Glutamine	115	59
2	0	0·160	—	62	—
	15	0·155	Glutamate	80	18
	15	0·157	Glutamine	107	45
	30	0·163	Glutamate	131	69
	30	0·154	Glutamine	124	62

(Krebs, 1948). When glutamate was the substrate in the medium over 90% of the total glutamate in the tissue was present as glutamic acid, whilst with glutamine as substrate 45% of the total glutamate in the slice was present as glutamine. Hence both forms can enter the tissue.

Anaerobic experiments. Anaerobically glucose partly prevented the fall in the concentration of glutamate which occurred when glutamate or glutamine were the only substrates in the medium (Table 6), and in some experiments (Exp. 3) there was even a small increase of the tissue glutamate in the presence of glucose. This increase was greater with glutamine than with glutamate in the medium. Adenosinetriphosphate and phosphoglycerate had no appreciable effect on the accumulation of glutamate. In these experiments glycolysis was also measured and it was noted that adenosinetriphosphate removed the glutamate inhibition (Weil-Malherbe, 1938) of glycolysis. In Exp. 1 of Table 6, for example, $Q_{\text{lactic acid}}$ was 6·4 in the presence of glucose and glutamate and 16·0 when adenosinetriphosphate was also added. The fact that addition of adenosinetriphosphate accelerates glycolysis may be taken to indicate that, in the presence of glutamate, adenosinetriphosphate is a limiting factor in glycolysis and it would follow that glutamate reduces the concentration of adenosinetriphosphate. This might be the result of the formation of glutamyl-γ-phosphate, a reaction postulated by Elliott (1948*a*, *b*) as a step in the synthesis of glutamine in brain. Addition of adenosinetriphosphate would overcome the inhibition of glycolysis by restoring the original concentration of this substance in the tissue. This hypothesis accounts for the inhibition of glycolysis by glutamate, which was previously unexplained, and for the abolition of the inhibition by adenosinetriphosphate.

Effects of inhibitors. Two kinds of effect, that on the concentration of glutamate in the tissue and that on the metabolic utilization of glutamate, were investigated. Guinea-pig brain cortex served as material and in all experiments the medium contained 0·02 M-glucose and 0·01 M-L-glutamate. The period of incubation was 40 min. DL-Methioninesulphoxide (0·02 M), sodium penicillin (0·1%) and strychnine (0·0025 M) had no effect. Sodium fluoride (0·02 M) and phlorrhizin (0·01 M) caused a fall in the tissue concentration of glutamate and in its utilization. Malonate (0·02 M) reduced accumulation a little and inhibited almost completely the metabolic utilization. In the presence of crystal violet (0·01%), 2:4-dinitrophenol (0·001 M) and iodoacetate (0·001 M) the utilization was not affected, but the accumulation was reduced in that the tissue concentration remained almost stationary, i.e. no fall occurred and no storage beyond the initial level.

To test whether D-glutamate has inhibitory effects DL- and L-glutamate were compared. Whilst accumulation of L-glutamate in the tissue was practically unaltered in the presence of DL-glutamate, much less, if any, L-glutamate appeared to have been metabolized. However, an analysis of this effect suggested that the apparent inhibition of L-glutamate removal by D-glutamate was due to a conversion of D-glutamate into L-glutamate by brain tissue. For example, on incubation (40 min.) of 155 mg. slices (fresh weight) with 0·01 M-D-glutamate and 0·02 M-glucose, an increase of 12 μl. of L-glutamate was found in the slices and of 38 μl. in the

Table 6. *Accumulation of* L-*glutamate in slices of brain cortex under anaerobic conditions*

(0·02 M-Glucose; 0·01 M-glutamate or glutamine; 0·002 M-adenosinetriphosphate; 0·02 M-DL-phosphoglycerate. Bicarbonate saline used in Exp. 1; phosphate saline in the other experiments. Incubation 30 min. in Exps. 1, 3 and 4; 40 min. in Exp. 2.)

Exp. no.	Substrate added	Initial wt. of slices (g.)	Amount of glutamate found in slices: Initial (μl.)	After incubation: Total (μl.)	After incubation: Change (μl.)
1	Glutamate; glucose	0·147	42	33	− 9
	Glutamate; glucose; adenosinetriphosphate; phosphoglycerate	0·155	44	38	− 6
	Glutamate; glucose; adenosinetriphosphate	0·161	46	38	− 8
	Glutamate; glucose; phosphoglycerate	0·145	41	41	0
2	Glutamate	0·101	42	15	−27
	Glutamine	0·100	42	21	−21
	Glutamine; glucose	0·101	42	28	−14
	Glutamine; adenosinetriphosphate	0·107	45	21	−24
	Glutamine; glucose; adenosinetriphosphate	0·100	42	27	−15
3	Glutamate; glucose	0·158	48	53	+ 5
	Glutamine; glucose	0·163	50	69	+19
4	Glutamate; glucose	0·164	58	46	−12
	Glutamine; glucose	0·168	59	61	+ 2

medium. No increase occurred in the absence of D-glutamate and it is therefore very probable that the L-glutamate formed had arisen from D-glutamate. Weil-Malherbe (1936) and Edlbacher & Wiss (1944) have shown that brain tissue can deaminate D-amino-acids, and deamination is presumably the primary step in the conversion of the D- into the L-form. This view is supported by the observation that α-ketoglutarate and ammonia yield L-glutamate in brain slices at a sufficient rate (Weil-Malherbe, 1936).

Accumulation of glutamate in tissues other than brain. Experiments similar to those on brain were carried out on nine other tissues (Table 7). Six of these, when suspended in saline containing L-glutamate and glucose, showed no increase in their glutamate concentration unless the concentration of glutamate in the medium was higher than the original concentration in the tissue. If a substrate for respiration was present, however, these tissues roughly maintained their original concentration of glutamate even when no glutamate was added to the medium. The six tissues were testis (rat), kidney medulla (guinea pig), liver (guinea pig), wall of the small intestine (rat), choroid plexus (sheep) and amnion (guinea pig). Records of the experiments on these tissues are not included in Table 7 except for one on guinea-pig liver. It was thought that the failure of this tissue to accumulate glutamate might possibly be due to the relatively low rate of penetration of glutamate (see Krebs, Eggleston & Hems, 1948), but this was not confirmed by the results of

Table 7. *Accumulation of* L-*glutamate by various tissues*

(Initial concentration of added substrates 0·02 M except where otherwise stated.)

Tissue	Substrates added	Period of incubation (min.)	Weight of tissue (g.)	Glutamate found in tissue (μl.)	Change of glutamate in medium (μl.)	Glutamate metabolized (μl.)	Final concentration of glutamate: Tissue (μl./g.)	Final concentration of glutamate: Medium (μl./ml.)
Kidney cortex, guinea pig	None	0	0·153	18	—	—	—	—
	None	60	0·152	11	+ 27	+ 20	72	7
	Glutamate (0·01 M)	60	0·146	61	−137	− 93	416	190
	Glutamate (0·01 M); glucose	60	0·153	63	−170	−125	412	182
Kidney cortex, guinea pig	None	0	0·131	19	—	—	—	—
	None	30	0·140	10	+ 23	+ 14	72	6
	Glutamate (0·01 M); glucose	30	0·155	44	− 94	− 69	283	188
Kidney cortex, guinea pig	None	0	0·154	22	—	—	—	—
	Glutamate (0·01 M)	40	0·148	46	− 94	− 70	313	189
	Glutamate (0·01 M); glucose	40	0·150	45	− 84	− 59	298	191
	Glutamate (0·01 M); L-lactate	40	0·147	38	− 52	− 36	258	199
Lung, guinea pig	None	0	0·180	23	—	—	—	—
	None	40	0·160	19	+ 21	+ 20	120	5
	Glutamate (0·003 M)	40	0·164	24	− 33	− 31	147	70
	Glutamate (0·003 M); glucose	40	0·173	22	− 22	− 22	127	73
	Glucose	40	0·161	15	− 10	+ 7	93	3
Lung, guinea pig	None	0	0·139	16	—	—	—	—
	None	40	0·151	12	+ 14	+ 10	80	3
	Glutamate (0·005 M); glucose	40	0·151	33	+ 40	+ 25	219	109
Spleen, guinea pig	None	0	0·103	7	—	—	—	—
	None	40	0·092	13	+ 14	+ 6	140	4
	Glutamate (0·003 M)	40	0·104	20	− 17	− 4	192	74
	Glutamate (0·003 M); glucose	40	0·112	20	− 14	− 3	178	75
	Glucose	40	0·106	22	− 23	+ 9	207	6
Liver, guinea pig	None	0	0·159	33	—	—	—	—
	None	60	0·161	10	+ 63	+ 40	62	16
	Glutamate (0·01 M)	60	0·161	26	−128	−135	161	192
	Glutamate (0·01 M); glucose	60	0·165	28	−117	−122	170	195
	Glucose	60	0·163	8	+ 37	+ 12	49	9
	Citrate; NH_4Cl (0·005 M)	60	0·165	20	+195	+182	121	49
	NH_4Cl (0·005 M)	60	0·164	7	+ 42	+ 16	43	11
Chorion, guinea pig	None	0	0·206	11	—	—	—	—
	Glutamate (0·01 M)	40	0·223	81	− 93	− 23	360	193
	Glutamate (0·01 M); glucose	40	0·207	76	− 85	− 19	367	195
Chorion, guinea pig	None	0	0·317	29	—	—	—	—
	None	40	0·333	21	+ 30	+ 21	62	8
	Glutamate (0·005 M)	40	0·294	64	− 73	− 39	217	90
	Glutamate (0·005 M); glucose	40	0·316	74	− 62	− 17	234	93

an experiment in which citrate and ammonium chloride were substrates (Table 7). Although glutamate was rapidly synthesized within the tissue (as indicated by its appearance in the medium) the concentration of glutamate in the tissue remained low.

Four guinea-pig tissues, kidney cortex, spleen, lung and chorion, gave results similar to those obtained with brain in that accumulation of glutamate occurred against a concentration gradient. Examples are given in Table 7. In kidney and chorion the concentration reached was greater than in lung and spleen, but in none of the four tissues did the glutamate concentration reach the high levels which were found in brain cortex. The addition of glucose to the medium did not affect accumulation in the four tissues.

A common feature of all the tissues examined is the ability to maintain a glutamate level above that of the medium if the concentration in the medium is low, i.e. similar to that found in blood plasma. The tissues differ with respect to the final level to which they can concentrate glutamate. Testis, kidney medulla, liver, intestinal wall, choroid plexus and amnion appear to be 'saturated' with glutamate at the start; they cannot increase the initial concentration. Brain, kidney cortex, chorion, lung and spleen are able to accumulate glutamate above the starting level.

The foetal membranes and placenta were examined as examples of growing tissues. They came from guinea pigs at approximately the 45th day of gestation. The results obtained with these materials revealed no correlation between accumulation of glutamate in the tissue and rate of growth.

In several experiments a small but definite amount of glutamate was formed on incubation of tissue when no glutamate was added to the medium (indicated in Table 7 by the positive signs in the column 'glutamate metabolized'). Most probably this glutamate was derived from proteins and peptides by hydrolysis.

Table 8. *Swelling of guinea-pig tissues in isotonic saline under aerobic and anaerobic conditions*

(Phosphate saline; 40 min. incubation; 0·02 M-glucose.)

	Change in wt. (% of initial wt.)	
Tissue	O_2	N_2
Brain cortex	+26	+52
Kidney cortex	0	+52
Liver	+14	+66
Lung	+ 1	+12
Spleen	−17	+ 4

Note on swelling of tissues in isotonic saline media. Reference has already been made in the Experimental section to the swelling of the tissue observed in the present experiments. Outstanding among the observations recorded was the fact that the weight increase found after anaerobic incubation was consistently greater than that after aerobic incubation (Table 8). As the amount of medium adherent to the incubated slices must have been approximately the same for both conditions, the differences in weight increase can only have been due to different degrees of swelling. Evidently respiration is an important factor in controlling the fluid uptake of the tissues.

DISCUSSION

Tissue slices as material for the study of permeability and storage. The experiments reported in this paper show that a mechanism is operative in slices of brain cortex and other tissues which transports glutamic acid from the medium into the tissue against a concentration gradient. In guinea-pig brain cortex the transfer stopped when the difference between the internal and external concentrations was about 0·02 M. Within certain limits this difference was independent of the absolute level of the external concentration. Tissue slices thus afford a system in which the mechanisms responsible for the permeability of the tissue and for the active storage of substances within the cell can be investigated under controlled conditions.

Sources of energy. The accumulation of glutamate against a concentration gradient is necessarily dependent on a source of energy. This accounts for the fact that substrates which can yield energy must be present if accumulation is to take place, and that inhibitors which interfere with the production of energy, such as sodium fluoride, also interfere with the accumulation of glutamate. Of the various substrates of oxidation, glucose was the most effective in brain, which is remarkable because lactate and pyruvate usually give rather higher Q_{O_2} values than glucose (see Elliott, Greig & Benoy, 1937). Glucose was less effective anaerobically than aerobically; it maintained the initial level but failed to support accumulation. The observation that adenosinetriphosphate had no effect should not be taken as evidence against the participation of energy-rich phosphate bonds in the energy transfer.

Energy requirements of brain. Brain, without doing external work, requires a continuous supply of energy, and it has been said that this energy is required to maintain a thermodynamically unstable structure. Few examples have been offered defining precisely in which respect the 'structure' of the tissue is 'unstable'. A high concentration of a substance in cells whose walls are permeable to the substance may be looked upon as a sign of an unstable structure. Glutamate in a concentration much above that of the blood plasma is in this sense a component of the 'structure' of brain which the forces of diffusion

tend to disintegrate. Energy must therefore continuously be added to maintain the 'structure'.

Permeability and metabolism. The present experiments provide a new demonstration of the influence of metabolic activities on cell permeability. As long as brain tissue is supplied with glucose, and the enzyme systems concerned with the energy supply from the glucose are intact, glutamate appears to permeate solely from the medium into the tissue and not in the reverse direction. But if the production of energy stops glutamate migrates from tissue to medium. Analogous observations have been recorded by Harris (1941), Wilbrandt (1940), and Maizels (1948), who studied the permeability of red blood cells for inorganic ions. Normal red cells seem to be impermeable to cations but when the rate of the energy-giving reactions of erythrocytes is lowered, either by chilling, or by inhibitors such as fluoride, or by lack of glucose, potassium and sodium diffuse through the cell wall according to the diffusion gradient. Restoration of the energy-giving reactions leads to redistribution of the cations towards their physiological state.

These observations, as well as those on swelling, make it clear that in the cases studied the dominating factor in cell permeability is not any inherent and constant physical property of the cell wall, like porosity or a lipid-sieve structure, but a mechanism dependent on the supply of energy. When this mechanism breaks down permeability changes and purely physical properties of the system come to the fore. It has of course always been appreciated that the supply of energy is an essential factor when substances are transported through cell walls in the processes of secretion and absorption. Hill (1931) and Krogh (1946) have already discussed the extension of this concept to the permeability of body cells in general, in particular also to the apparent absence of transport through the cell wall of cell constituents.

It would be premature to speculate on the nature of the mechanism by which energy is used to maintain concentration gradients. An experimental approach to the problem might be based on the hypothesis that electrical energy is the immediate form of energy which controls the movement of ions.

Accumulation of metabolites in tissues as a source of error in quantitative experiments. It has been customary to measure rates of metabolism in tissue slices by estimating the quantities of the metabolites removed from the medium. It is evident that errors arise if the substances removed are not metabolized but stored in the tissue, or conversely, if the tissue releases substances into the medium from accumulated stores. The measurement of the true metabolic rates requires in such cases the inclusion of the tissue in the analysis.

Accumulation of glutamate in micro-organisms. Reference should be made to the work of Gale on Gram-positive bacteria and of Taylor on yeast (reviewed by Gale, 1948) concerning the relations between extracellular and intracellular concentrations of glutamate and other amino-acids. The phenomenon of amino-acid accumulation is in general very similar in certain micro-organisms and in animal tissues, but details such as the quantitative relations between internal and external concentration, the effects of inhibitors and of substrates show many differences. This is hardly surprising as the micro-organisms themselves show great variations from species to species and within the same species with varying ages of the culture.

SUMMARY

1. Slices of guinea-pig brain cortex, when suspended aerobically in a saline medium containing glucose and L-glutamate, accumulate L-glutamate within the tissue. The transport of glutamate into the tissue occurs against a concentration gradient. Conditions for studying this phenomenon have been elaborated.

2. Transport of L-glutamate into the tissue stopped when the difference between the concentrations in the tissue and in the medium was about 0·02M.

3. The concentration of L-glutamate in the tissue fell when glucose was omitted from the medium. Substrates which supported accumulation of glutamate were glucose, fructose, L-lactate and pyruvate, but in most experiments glucose was more effective than the other substrates. Succinate, α-glycerophosphate or excess of glutamate did not prevent the fall of the initial glutamate concentration in the brain, although these substances are readily oxidized by this tissue.

4. Whilst brain slices retained and accumulated L-glutamate, the very much larger molecules of the glutamic-aspartic transaminase readily diffused from the slices into the medium.

5. L-Glutamine was taken up by brain tissue a little more rapidly than L-glutamate, but the final concentrations reached were about the same.

6. Anaerobically no appreciable accumulation occurred, but glucose delayed the fall of the initial concentration of glutamate in the tissue.

7. D-Glutamate was converted into L-glutamate by brain slices.

8. Adenosinetriphosphate abolished the inhibition of brain glycolysis by glutamate. It is suggested that the glutamate inhibition is due to an interaction between glutamate and adenosinetriphosphate to form glutamyl-γ-phosphate and that the removal of adenosinetriphosphate by this reaction is responsible for the slowing down of glycolysis.

9. Accumulation of glutamate also occurred in isolated kidney cortex, spleen, lung and chorion. Six other tissues examined maintained a concentration gradient between tissue and medium, but did not increase the glutamate concentration above the original level.

10. It is pointed out that tissue slices afford a system in which problems of permeability and storage can be studied under controlled conditions. The permeability of brain tissue for glutamate is controlled by energy-giving metabolic processes.

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Displacement Chromatography on Synthetic Ion-exchange Resins

1. SEPARATION OF ORGANIC BASES AND AMINO-ACIDS USING CATION-EXCHANGE RESINS

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The work described in Parts 1 and 2 of this communication represents an attempt to apply the principles of the flowing chromatogram to the separation of organic electrolytes on synthetic ion-exchange resins. The intention has been to develop the method for application to biochemical preparations on a fairly large scale, since the special properties of the resins render them particularly suitable for this purpose. The possibility of using an adaptation of the method for the quantitative analysis of mixtures of bases has not been considered here.

A preliminary account of this work was published in 1947 (Bendall, Partridge & Westall, 1947; Partridge, Westall & Bendall, 1947), and soon afterwards an analytical procedure based on similar principles was published by Drake (1947). Both methods are based upon the techniques described by Tiselius (1943) and Claesson (1946), who applied the principle of 'displacement development' to separations on adsorption columns of carbon or alumina and developed a quantitative analytical technique suitable for application to small quantities of material.

The advent of the sulphonated cation-exchange resins has made it possible, for the first time, to effect the quantitative adsorption of cations from solutions of neutral salts, whilst the development of ion exchangers of the polyamine-formaldehyde type has allowed the removal from the resulting solution of the anions liberated in the form of free acids. Many applications of the newer synthetic resins for the purpose of removing undesired electrolytes from biological solutions have been reported (cf. Platt & Glock, 1943; Partridge, 1948), but the technique to be described here seeks to take the use of ion-exchange resins a stage further by effecting the chromatographic fractionation of both the acids and the bases present in the solution with a view to the eventual isolation in a pure condition of such components as may be of interest.

In order to make the principles of the technique clear from the outset, the essentials of the method (as particularly applied to the fractionation of a mixture of bases) may be stated briefly as follows. A solution of the mixture of bases to be separated is introduced on to a column containing a cation-exchange reagent, and the components of the mixture are displaced down the column by application of a solution of a base that is more strongly adsorbed than any of the bases in the mixture. Once a flowing equilibrium has been established, the less strongly adsorbed bases are successively displaced by those having a stronger affinity for the ion exchanger, and the components of the mixture pass down the column in a series of discrete bands, finally flowing from it in an order dependent upon the strength with which they are adsorbed.

In the experiments described in Part 1 of this series the cation exchanger 'Zeo-Karb 215' was used. This resin may be obtained from the Permutit Co. Ltd., London.

METHODS

Since in general the bands formed by electrolytes adsorbed on columns packed with ion-exchange resins are not clearly visible, the separations that take place on the column must be followed by measuring changes in composition of the effluent, and means are required whereby qualitative and quantitative information may be obtained continuously as the solution flows from the column. Various methods of following changes in the physical properties of the effluent in flowing chromatograms have been suggested (Claesson, 1946), but the three methods found most suitable for the purpose of the present work are: (*a*) continuous measurement of electrical conductivity; (*b*) continuous measurement of hydrogen-ion concentration; (*c*) titration of small successive volumes of effluent.

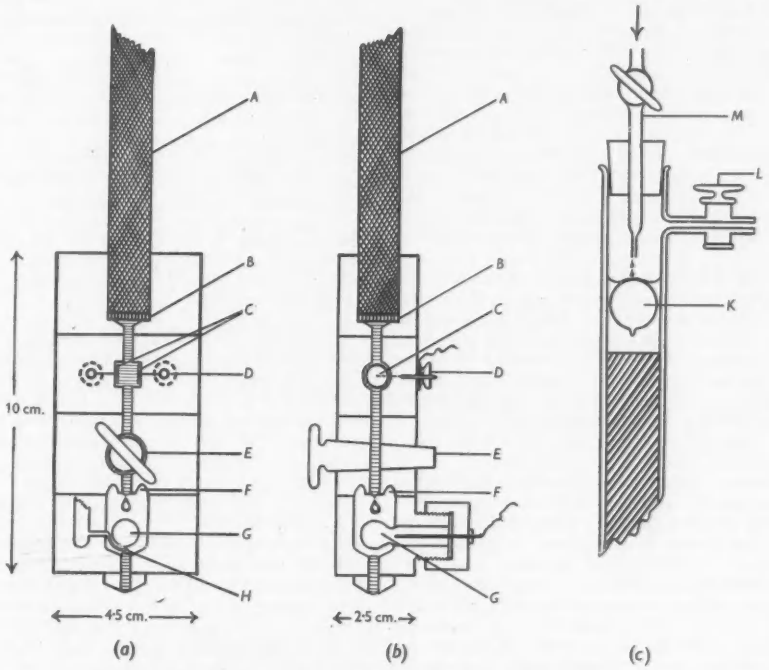

Fig. 1. Column assembly. (*a*) Front elevation; (*b*) side elevation; (*c*) upper portion of column showing inlet and float.

To these three physical methods may be added a fourth that depends specifically on the chemical constitution of the solute. In the separation of mixtures of amino-acids it is usually of advantage to take a large number of samples from the effluent at fixed intervals, and to carry out the qualitative analysis of each sample by use of the filter-paper chromatogram of Consden, Gordon & Martin (1944).

Measurement of pH *and electrical conductivity.* Fig. 1 illustrates the form of apparatus adopted for small-scale laboratory investigations. The units of the assembly were interchangeable, and consisted of blocks of 'Perspex' which were accurately machined and fitted one above the other in a frame constructed from the same material. The assembly comprised: (1) a series of interchangeable blocks designed to carry columns of varying diameter, (2) a block carrying the

conductivity cell, (3) a block carrying a tap for regulating the rate of flow of the solution, (4) a block carrying the glass-electrode cell. In use the blocks were sealed together by means of a thin film of soft petroleum jelly.

The column (*A*) was of glass and was closed by a perforated disk of Perspex (*B*) above which was a thin uniform layer of glass wool. The black platinum electrodes (*C*) were 1 cm. in diameter, and were accurately fitted into the flat faces of a cylindrical cavity so that only one face of each electrode was exposed to the solution. The electrodes were connected by platinum wire to the terminals (*D*) mounted on the back of the Perspex block. Regulation of the rate of flow of the solution was carried out by means of a tapered tap (*E*) made from Perspex and lubricated with vaseline. The glass electrode (*G*) consisted of a bulb blown on the end of a tube of soft glass (Corning, 015) and was mounted in a cylindrical cavity (diameter 15 mm.) in the lower block. The tube carrying the bulb (which in use was filled with 0·1 N-HCl) was sealed at the open end by means of a threaded Perspex collar provided with a screw cap and a rubber gasket through which a silver wire passed to the terminal. The KCl bridge connexion was made by leading a fine glass capillary (*H*) to a position immediately below the bulb.

To avoid the possibility of electrical leakage from the conductivity cell to the glass-electrode cell the liquid level in the latter was not allowed to rise above the centre of the bulb, and an annular recess was provided in the base of the tap section (shown at *F* in Fig. 1) in order to induce the liquid to fall on the electrode bulb in the form of small drops.

The resistance of the conductivity cell was measured by means of a 'Mullard' bridge energized by a 1000-cycle oscillator, while pH was recorded as millivolt (mV.) readings by means of a 'Unicam' potentiometer.

Determination of break-through volumes. When a solution of a base is passed through a cation-exchange column a 'band' forms on the upper part of the column and the effluent at first consists of pure solvent only. The region of partial saturation in front of the band is called the 'boundary' of the band, and under varying conditions the boundary may be sharp or more or less diffuse. As further solution is applied the band extends downwards, and when the boundary reaches the bottom of the column the concentration of the solute in the effluent rapidly increases until finally the solution flowing out has the same concentration as that flowing in. At this point the whole of the column material is in equilibrium with the inflowing solution.

The changes in concentration of the effluent may usually be measured by direct titration, and Fig. 2 shows a volume-concentration curve obtained in this way. The volume V_i (Fig. 2) represents the volume of water contained in the apparatus before the experiment begins and includes the water enclosed within and between the particles of resin. To obtain a value for V_i, the apparatus is first weighed empty. The column is then packed, and the water level adjusted to a fixed mark on the column after which the apparatus is reweighed. The weight of the dry resin is then subtracted from the difference in weights so obtained, thus giving the total weight of water initially present in the apparatus. V_0 (Fig. 2) is the volume of effluent measured to the point at which the solute first breaks through, while V_{90} is the volume of effluent measured to the point at which the outflowing solution reaches 90 % of the concentration of the solution supplied to the column. V_e represents the break-through volume calculated on the assumption that the boundary is perfectly sharp, and is derived by fixing the position of the line *DB* such that the area *ABC* equals the area *ADE*. Thus the weight of solute adsorbed by the resin is given by $C \times V_e$, where C is the concentration of the inflowing solute. Unless otherwise stated, the values given for V_0, V_{90} and V_e in this paper are corrected for the small volume V_i.

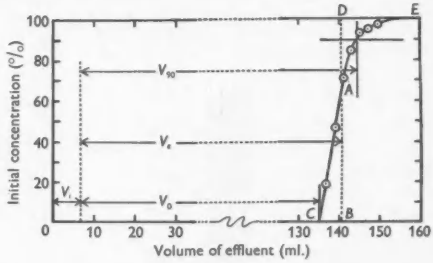

Fig. 2. Volume-concentration curve for effluent from 0·042 N-NH_3 solution on 'Zeo-Karb 215' (1·45 g., mesh size 120–150) contained in a column 8·5 mm. in diameter (volume axis shortened).

Construction and packing of the columns. Faults in the construction or packing of columns may often be recognized from the shape of volume-concentration curves of the type given in Fig. 2, which shows the typical curve obtained from a well-packed column. With faulty columns $V_{90} - V_0$ is increased and the curve may be markedly sigmoid in shape or may show the presence of a 'foot' due to distortion of the boundary as it progresses down the column.

Various methods of packing the column were investigated. The technique usual in adsorption chromatography, in which the columns are packed dry and tamped at frequent intervals, was found to be unsuitable for use with ion-exchange resins, since the expansion that takes place as the resin swells in water tends to disturb the uniformity of distribution of the particles. The method finally adopted as a standard procedure for packing the cation-exchange resin 'Zeo-Karb 215' was as follows. The resin, after grinding in a dry condition in a hammer mill, was graded by sieving. The graded resin was allowed to swell in 5 N-HCl overnight, and was then treated alternately two or three times with 2 N-HCl and 2 N-NaOH by sedimentation in a beaker. During this process a certain amount of fine material that gave rise to a cloudy suspension in water was rejected, and the final supernatant fluid was clear and colourless. The resin was then washed with distilled water until substantially free from HCl and dried at room temperature on open trays. A water-content determination was made on the sample of resin by drying to constant weight over P_2O_5 *in vacuo* at 80°, and the value was used to calculate the true dry weight of the resin introduced into the column. A weighed quantity of the resin was allowed to swell in water and was completely transferred in small portions to the column, which was previously filled with water. As the particles of resin fell to the bottom of the column, water was allowed to drip from the lower tap in order to avoid the disturbance caused by the upward displacement of water by the falling resin. In this way the column was built up as a series of flat

laminae and no attempt was made to consolidate it by tamping or tapping. The column, once made, was not allowed to drain dry and was always kept with a small depth of water above the resin. Before use the column was treated two or three times alternately with 2N-HCl and N-NH_3, and after finally activating with 2N-HCl, was washed with distilled water until the conductivity of the effluent reached a steady value.

The design of the column has a marked influence on the sharpness of the boundaries obtained in separation experiments, and particular attention should be paid to the construction of the lower orifice. The type of construction shown in Fig. 1 (*a* and *b*), which embodies a perforated plate (*B*), has given good results in practice, and was designed to ensure that the boundary leaves the column in a uniform manner and is not impeded or distorted by a dead space at the shoulders of the outlet tube.

The arrangement of the inflow to the column is also important. The liquid entering the column should in no case be allowed to flow down the side since this usually gives rise to a sloping boundary which, once it is formed, frequently persists throughout the experiment. Fig. 1 (*c*) shows an arrangement that has proved satisfactory. The water level is maintained a little above the top of the resin and a small spherical glass float (*K*) is introduced in order to break the force of the falling drops of solution. The solution is introduced from a separating funnel through a glass tube (*M*) which is drawn out to a capillary to reduce the size of the drops. The water level in the column is maintained at a fixed position throughout the experiment by manipulation of the side tap (*L*).

RESULTS

Construction of 'retention isotherms'. The retention of bases and ampholytes on the cation-exchange column may be expressed quantitatively in the form of an 'isotherm' in which the amount of substance adsorbed/g. of adsorbent is plotted against the concentration of the solution. Such curves may be plotted from measurements of V_e at various concentrations of the solute, using columns containing a known weight of resin. If ϵ is the amount of solute adsorbed (millimol./g. dry wt. of resin), W the dry weight of the resin, and c the concentration (millimol./ml.) of the solution flowing into the column, then

$$\epsilon = \frac{V_e \times c}{W}. \qquad \text{(i)}$$

Fig. 3 shows the retention curves obtained by this method for ammonia and a number of amino-acids using 'Zeo-Karb 215'.

The curves given by the stronger bases show considerable deviation from the Langmuir adsorption equation (Langmuir, 1918). This behaviour is to be expected since in experiments of the kind illustrated in Fig. 3, in which decreasing concentrations of a base are applied to the column, there are two effective variables, the concentration of the cation and the concentration of hydrogen ions. In addition, since the resin used is of the sulphonated phenol-formaldehyde type, it possesses weakly acid phenolic hydroxyl groupings which accept cations from the more strongly alkaline solutions but are inert under conditions of lower pH.

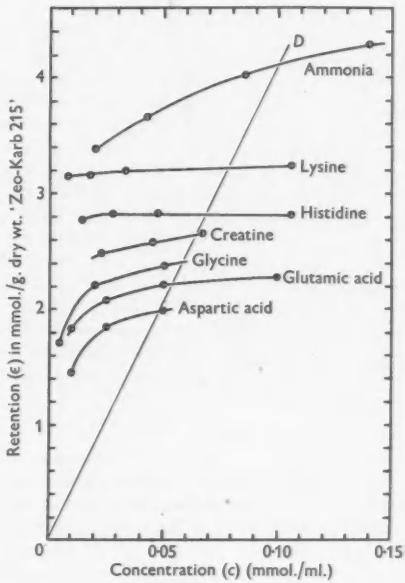

Fig. 3. The retention of ammonia and a number of amino-acids on 'Zeo-Karb 215' (80–100 mesh). The experiments were carried out with a column 8·5 mm. in diameter containing 1·44 g. 'Zeo-Karb' (dry wt.).

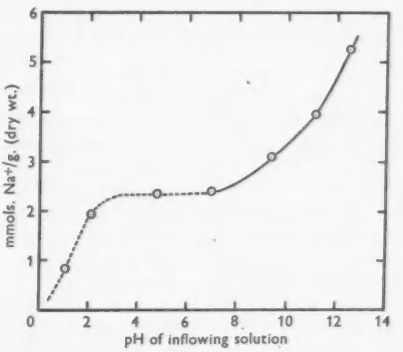

Fig. 4. Adsorption of Na^+ from a range of buffers containing NaOH (0·053N) adjusted to pH 12·1–1·1 by the addition of acids.

The effect of the hydrogen-ion concentration of the inflowing solution is illustrated in Fig. 4 in which ϵ is plotted for the adsorption of sodium ions from a range of buffer solutions each containing

NaOH (0·053N). The solutions were adjusted to pH 12·5–1·1 by the addition of various acids. The buffer solutions were as follows: pH 12·5–9·35, NaOH + boric acid; pH 6·9, NaOH + NaH_2PO_4; pH 4·7, NaOH + acetic acid; pH 2·1–1·1, NaOH + HCl. The resulting curve of ϵ values (Fig. 4) is plainly a titration curve of the resin, but differs from the usual form of such curves in that the concentration of sodium ions in the system has been maintained at a constant value. The shape of the curve shows that there are two main absorbing groups: a strongly acid group (approx. pK 1·5) which is presumably the sulphonic acid radical, and a series of weakly acid groups of pK > 10 which may be identified with phenolic residues. These latter may be modified by the formation of quinonoid structures, and for that reason may be more strongly acid in reaction than is the case with simple phenols.

In Fig. 4 the range pH 0–3 represents the region over which the sulphonic acid radicals of the resin are being titrated by the base and the reaction may be represented by

$$R\mathrm{H} + \mathrm{Na}^+ \leftrightharpoons R^- \ldots \mathrm{Na}^+ + \mathrm{H}^+,$$

where RH is the hydrogen form of the resin. (The symbol $R^- \ldots \mathrm{Na}^+$ is used to represent the dissociated resin salt in which movement of the cation Na^+ is restricted by the effect of the negative charges on the resin.) From this

$$K = \frac{[R^- \ldots \mathrm{Na}^+]\,[\mathrm{H}^+]}{[R\mathrm{H}]\,[\mathrm{Na}^+]}. \qquad \text{(ii)}$$

The term $\dfrac{[R^- \ldots \mathrm{Na}^+]}{[R\mathrm{H}]}$ may be replaced by $\dfrac{\epsilon}{(\epsilon_{\mathrm{sat.}} - \epsilon)}$, where $\epsilon_{\mathrm{sat.}}$ is the maximum adsorption of Na^+ in mmol./g. of resin (dry wt.) by the sulphonic acid residues of the resin. Thus

$$K = \frac{\epsilon}{(\epsilon_{\mathrm{sat.}} - \epsilon)} \frac{[\mathrm{H}^+]}{[\mathrm{Na}^+]}. \qquad \text{(iii)}$$

A value for $\epsilon_{\mathrm{sat.}}$ is given by the ordinate of the horizontal section (pH 5–6) of the experimental curve (Fig. 4), and K may be calculated by use of equation (iii) from any experimental point on the curve in the range pH 0–3. The dotted portion of the curve in Fig. 4 (between pH 0 and 7) was calculated from (iii) taking $\epsilon_{\mathrm{sat.}} = 2{\cdot}35$ and $K = 0{\cdot}82$.

Over the region pH 3–7, ϵ approaches $\epsilon_{\mathrm{sat.}}$ and the system becomes insensitive to changes in $[\mathrm{H}^+]$. In this condition the resin acid may be regarded as fully ionized with respect to the sulphonic acid groups and the reaction with a cation B^+ may be written

$$R^- \ldots \mathrm{H}^+ + B^+ \leftrightharpoons R^- \ldots B^+ + \mathrm{H}^+.$$

In this system the negatively charged centres R^- are restricted in movement by attachment to the polymer, and a condition approaching electrical neutrality is maintained by the orientation of cations, hydrogen ions or the ions of ampholytes about the negative centres. The adsorption of a cation B^+ will therefore be affected by conditions relating to the size and charge of the cation and its activity in the external solution. Where anions are present in the external solution they will also be present to some extent in the resin gel and 'adsorption' of anions by the resin may be considerable where B^+ is polyvalent.

By application of the Donnan equation to the equilibrium between the resin gel and the external solution in the system

$$R\begin{cases}\mathrm{Na}_R^+ \\ \mathrm{H}_R^+\end{cases} \quad \mathrm{Cl}_R^- \quad \Big\| \quad \begin{matrix}\mathrm{Na}_s^+ \\ \mathrm{H}_s^+\end{matrix} \quad \mathrm{Cl}_s^-$$

Bauman & Eichhorn (1947) derive the relation

$$\frac{[\mathrm{Na}_R].[\mathrm{H}_s]}{[\mathrm{H}_R].[\mathrm{Na}_s]} = k = \frac{f\mathrm{H}_R}{f\mathrm{Na}_R}, \qquad \text{(iv)}$$

where $[\mathrm{Na}_R]$ and $[\mathrm{Na}_s]$ are the concentrations of Na^+ in the resin phase and the solution phase, respectively, and $[\mathrm{H}_R]$, $[\mathrm{H}_s]$ are the corresponding concentrations of H^+. $f\mathrm{H}_R$ and $f\mathrm{Na}_R$ are the molar activity coefficients of H^+ and R^+ in the resin phase. Equation (iv) was shown to be valid for dilute solutions of salts of monovalent inorganic bases. From their experimental results Bauman & Eichhorn (1947) were led to the opinion that the ion-exchange equilibria between monovalent metallic cations on Dowex 50 (a resin that contains $-SO_3H$ as the only acidic radical) is determined simply by the differences in activity coefficients between the outside solution and the very highly concentrated solutions in the resin gel, without involving any specific affinity of $-SO_3H$ groups for one ion over another.

The exchange reaction taking place between two weak organic bases is, however, significantly different from that occurring between the salts of alkali metals, since in the former case one base may depress the dissociation of the other, and the cations of the stronger base will therefore be more available to satisfy the negatively charged sites on the resin. Thus in the special case of the exchange of organic cations between the resin and a solution of a weak base or amino-acid, the effect of the stronger base in suppressing the ionization of the weaker base in the liquid phase becomes an important consideration, and, as will be shown later, there is a strong presumption that this effect is a factor in determining the order of displacement of a series of organic bases and ampholytes.

The equation of Bauman & Eichhorn (iv) is of the same form as the mass action equation (iii) and may be reduced to

$$k = \frac{\epsilon}{\epsilon_{\mathrm{sat.}} - \epsilon} \times \frac{[\mathrm{H}_s]}{[B_s]}, \qquad \text{(v)}$$

where the two constants k and $\epsilon_{\mathrm{sat.}}$ refer specifically to the exchange of hydrogen ions for the cation B^+.

If the pH of the equilibrium solution is maintained at a constant value, $[H_s]$ may be neglected and equation (v) becomes

$$k' \epsilon_{sat.} - k'\epsilon = \frac{\epsilon}{[B_s]}. \qquad \text{(vi)}$$

This equation is of the same form as the 'adsorption isotherm' of Langmuir (1918), and describes the adsorption of cations by the resin acid from a solution of a base at constant pH. It should therefore be applicable to the adsorption of the acidic and neutral amino-acids since solutions of these show little pH change on dilution. The validity of equation (vi) for the adsorption of the amino-acids may be demonstrated by plotting the values of ϵ against $\epsilon/[B_s]$. From the data in Fig. 3 the plots for glutamic acid, aspartic acid and glycine fall on a series of straight lines at concentrations within the useful range.

The separation of mixtures of bases and ampholytes. Fig. 5 gives the results of a typical experiment in which a mixture of three amino-acids was separated by displacement with ammonia solution. In this experiment a mixture of aspartic acid, glycine and histidine was chosen for the reason that the isoelectric points of these amino-acids are well separated on the pH scale, and thus they show no tendency to form mixed bands on the column.

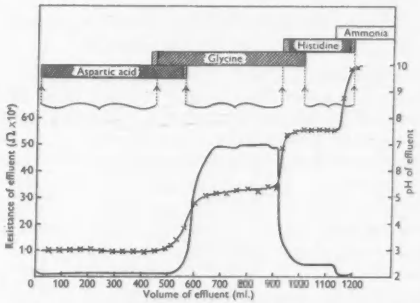
Ammonia
Histidine
Glycine
Aspartic acid
Resistance of effluent (Ω x 10³)
pH of effluent
Volume of effluent (ml.)
0 100 200 300 400 500 600 700 800 900 1000 1100 1200
10 20 30 40 50 60
2 3 4 5 6 7 8 9 10

Fig. 5. The separation of aspartic acid, glycine and histidine by displacement with 0·10N-NH_3. Smooth curve, resistance; ×—×, pH values.

The column used was 15 mm. in diameter and 400 mm. high. It contained 28 g. (dry wt.) of 'Zeo-Karb 215' which had previously been ground to 40–60 mesh/in. and prepared according to the directions aready given. An aqueous solution (1 l.) containing L-aspartic acid, 2·0 g., glycine, 2·0 g. and L-histidine monohydrochloride, 2·7 g. was passed through the column at a rate of 250 ml./hr., after which the column was washed with a further 500 ml. distilled water in order to ensure the removal of residual hydrochloric acid from the amino-acid hydrochloride.

Displacement was carried out with 0·10N-NH_3 (1210 ml.), which was introduced at a rate of 200 ml./hr., and the effluent was collected in 10 ml. fractions. The conductivity of the effluent was recorded continuously during the run, and the results are plotted in the form of a smooth curve in Fig. 5. The figure also shows pH readings determined on every fourth fraction after the run was complete.

It will be seen from the form of the curves that the course of the fractionation can be followed from the pH and conductivity measurements alone, but in order to provide a more sensitive check on the purity of the fractions, a drop of the solution was taken from each 10 ml. fraction and introduced on to a filter-paper chromatogram. This was irrigated with phenol and developed with ninhydrin according to the technique of Consden *et al.* (1944). The resulting chromatogram is represented diagrammatically in Fig. 5. The lightly hatched areas at the leading and trailing edges of the amino-acid bands represent the presence of the amino-acid as a trace only. It is clear from the diagram that the effluent collected in fractions 1–42 contained aspartic acid only, while those in fractions 56–92 and fractions 101–114 contained pure glycine and pure histidine respectively. Fractions 115–121 contained histidine and ammonia, but since the latter may readily be removed by evaporation under reduced pressure, the material was suitable for addition to the main histidine fraction.

Fractions 1–44, 56–92 and 101–121 were combined, and in each case the solution was evaporated to small bulk under reduced pressure. The concentrated solutions were then evaporated to dryness under vacuum. A colourless crystalline product was obtained in each case, in the yields given below; the purity of the fractions was confirmed by analysis for nitrogen.

	Yield (g.)	Yield (%)	N (%) (Found)	N (%) (Calc.)
Aspartic acid	1·63	81·5	10·5	10·5
Glycine	1·54	77·0	18·7	18·7
Histidine	1·77	80·5	27·0	27·1

The concentration of the solutes in the effluent solutions. For the purpose of designing a suitable fractionation procedure it may be necessary to calculate the concentration at which each component will be delivered from the column under given conditions. The graphical method of Claesson (1946) may be adopted for this purpose. In Fig. 3 a line $0D$ is drawn from the origin (0) to a point D on the isotherm of the displacement developer which represents the concentration of the developer to be applied. The point at which the line $0D$ cuts the isotherm of any component (A) gives the concentration at which the solution of A will flow from the column.

The gradient of $0D$ is $\Delta\epsilon/\Delta c$ and is a function of the break-through volume of the developer, thus

$$\frac{W\Delta\epsilon}{\Delta c}=\frac{W\epsilon_d}{C_d}=V_d, \qquad \text{(vii)}$$

where W is the weight of resin in the column and ϵ_d, C_d and V_d refer to the developer D. Similarly, for any other component A,

$$\frac{W\Delta\epsilon}{\Delta c}=\frac{W\epsilon_a}{C_a}=V_a, \qquad \text{(viii)}$$

where ϵ_a, C_a and V_a refer to the point of intersection of the line $0D$ with the isotherm of A. Thus the point of intersection determines the concentration of the component A such that $V_a=V_d$. From this it is clear that if the component A is displaced by component D (applied at concentration C_d) and the column is sufficiently long for the establishment of a flowing equilibrium, then A will flow from the column at the concentration C_a. If A is initially applied to the column at a concentration lower than C_a, then the band due to A contracts on application of the developer until the adsorbed solute reaches equilibrium with a solution of concentration C_a; similarly, if A is initially adsorbed from a concentration higher than C_a the band increases in width during development.

The argument given above is valid for a displacement experiment only in so far as the component is in the same ionic environment as prevails during the determination of the individual isotherms. This condition is most probably completely fulfilled where weak electrolytes are adsorbed from organic solvents by such reagents as carbon, but may not be fulfilled in practical experiments with ion exchangers. Nevertheless, where free bases such as ammonia, sodium hydroxide or barium hydroxide were used as displacement developers, the concentrations found by experiment showed good agreement with the calculated values. Displacement with solutions of salts, however, introduces the complication of an anion, and under such circumstances a calculation based upon the isotherms determined for the free bases is rendered valueless.

Where displacement experiments are carried out on a mixture of solutes it is important to ensure that the concentration at which the least soluble component is displaced does not greatly exceed its solubility in the solvent, for should this occur anomalous results may be obtained due to the formation of crystals in the column. However, in practice, it is often possible to displace a solute at a concentration slightly in excess of its solubility, and in this case crystals of the pure solute may appear in the fractions taken from the effluent. This effect was observed in the separation experiment illustrated in Fig. 5. In this experiment 0·10 N-ammonia was used for displacement, and crystals of aspartic acid appeared in the effluent solution soon after the fractions were collected.

The order of displacement. In displacement chromatograms carried out on adsorbents such as carbon, the order of displacement of a series of solutes is given directly by the relevant isotherms, the solutes that are adsorbed to a greater extent displacing those adsorbed less strongly (Claesson, 1946). Where ion exchangers are used this rule is not universally applicable, and anomalies are noted particularly where mixtures of monovalent bases, divalent bases and amino-acids are displaced from cation-exchange columns. Table 1 lists a number of bases and amino-acids in the order in which they are displaced from 'Zeo-Karb 215' and shows that this order does not follow exactly the order of decreasing values of ϵ. The brackets in the table include pairs of solutes that form mixed bands on the column.

If we consider a point on a column at the boundary between two bands, both solutes will be present in the liquid phase, and the stronger base will tend to suppress the ionization of the weaker. Thus the stronger base will be preferentially adsorbed, and for this reason it would be expected that the pH of the emerging solutions would give a more exact guide to the order of displacement than the values of ϵ, which are affected by the specific effects of the cations. The results given in Table 1 bear out this general rule; thus, although in general the values of ϵ and the pH of the emerging solutions run parallel, there are certain irregularities, and the order of displacement appears to be more accurately given by the order of pH. Subsequent experience has shown that the pH rule is useful for the purpose of predicting the expected position of bases and amino-acids in flowing chromatograms. The pH of a solu-

Table 1. *Retention data for a number of bases and ampholytes on 'Zeo-Karb 215'*

(The bases are given in the order of displacement, but the substances included in the brackets do not separate on the column. ϵ is the amount of solute (mmol.) retained by 1 g. of dry resin from a solution of concentration c (mmol./ml.) when this is passed continuously through a column of the resin.)

Substance	Equilibrium conc. (c) (mmol./ml.)	ϵ (mmol./g. dry wt.)	pH of emerging solution
{NaOH	0·071	4·77	12·9
{$Ba(OH)_2$	0·05	3·38	12·7
Ammonia	0·05	3·70	11·0
Lysine	0·05	3·20	9·7
Anserine	0·035	2·35	8·3
{Carnosine	0·035	2·35	8·2
{Creatinine	0·041	2·75	8·2
Creatine	0·05	2·60	7·0
Glycine	0·05	2·36	6·0
{Serine	0·05	2·26	5·1
{Glutamic acid	0·05	2·20	3·2
Aspartic acid	0·05	1·98	2·8

tion of an amino-acid, as it flows from a cation-exchange column, lies near its isoelectric point, provided that the mixture of solutes is displaced by a base and anions are excluded from the system.

The behaviour of serine presents an important exception to the pH rule since this amino-acid forms a mixed band with glutamic acid.

Factors influencing the width of the boundaries. Fig. 2 represents the variation in concentration of the solute as a band emerges from the column. The values for V_i, V_{90}, V_0 and V_e are readily determined experimentally, and it is clear that the expression $V_{90}-V_0$ gives an indication of the sharpness of the front leaving the column. The height (λ_{90}) of the cylinder of column material occupied by the boundary of the front is given by

$$\lambda_{90}=\frac{(V_{90}-V_0)}{V_e}L, \qquad \text{(ix)}$$

where L is the length of the column in cm. λ_{90} may be called the 'boundary width' and since $(V_{90}-V_0)/V_e$ is independent of the cross-sectional area of the column and varies inversely as its length, λ_{90} is independent of both the diameter and the length of the column. The value of λ_{90} is characteristic of the sample of resin, and is increased with increasing particle size and increasing rate of flow of solution (cf. Glueckauf & Coates, 1947). Since λ_{90} is dependent upon the rate at which equilibration is attained its value may be affected by variations in temperature and should be greater with solutes of high molecular weight. Measurements of λ_{90} are most easily carried out by examining the front given by a single basic component displacing H^+ from the ion exchanger. However, the boundary between two basic components may also be examined, and in those cases where the two components do not differ greatly in strength as bases the value of λ_{90} is frequently found to be very large. This subject has been discussed in relation to the shape of the two-component isotherm by Glueckauf (1947).

In Fig. 6 values of λ_{90} are given for a number of samples of 'Zeo-Karb 215' of different particle size which were graded by sieving in the dry state. The solution used for the determinations of $(V_{90}-V_0)/V_e$ was in each case 0·042N-NH_3, and all the results were obtained with 1·44 g. of resin (dry wt.) packed in a column 8·5 mm. in internal diameter. In the figure, λ_{90} has been plotted against the rate (S) at which the boundary travels down the column in cm./hr. S may be measured independently of the dimensions of the column, and for this reason it was used in preference to the rate of flow of the solution (v); S and v are related by the expression

$$S=L\frac{v}{V_e}, \qquad \text{(x)}$$

where v is expressed in ml./hr.

Fig. 6 gives a clear indication of the effect of increasing particle size on the width of the boundary obtained and also shows a rather smaller effect in the same direction due to increasing the rate of flow of the solution. The value of λ_{90} is clearly related to a power of the average particle diameter and, except at small values of $(V_{90}-V_0)$ is nearly proportional to the rate of flow of the solution. This is in general agreement with the predictions of Glueckauf (1947).

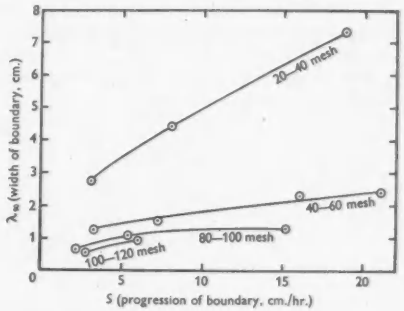

Fig. 6. The effect of particle size and rate of flow on the width of the boundary of an ammonia front. (NH_4^+ displacing H^+ from 'Zeo-Karb 215'.)

The determination of values for λ_{90} for a series of sieve-graded sizes offers a method for assessing the suitability of a resin for the purpose of displacement chromatography, and experience so far obtained indicates very large differences in λ_{90} for various resins. However, in comparing the boundaries given by two different resins for the same solute, the adsorptive capacity of the resins for the solute should be taken into account; if one of the resins adsorbs less of the solute, then a longer column can be used to handle a given weight of the solute and the width of the boundary that can be tolerated will increase proportionately. For this reason it may be preferable to compare the values of $\lambda_{90}\times\epsilon$ for a series of related resins of differing adsorption capacity, rather than the direct values of λ_{90}. The performance of 'Zeo-Karb 215' was excellent when compared with that of other resins investigated, and inspection of Fig. 6 shows that, with this resin, little advantage is obtained from grinding the resin to a particle size smaller than 80–100 mesh/in. For this reason 80–100 mesh powder has been adopted as standard for smaller columns, but since, for longer columns, there is advantage to be obtained from the higher rates of flow obtainable with larger particles, a powder sieving between 40–60 mesh has usually been used for experiments on a larger scale.

The values for λ_{90} given in Fig. 6 were obtained for ammonia solution, but parallel experiments carried out on more basic substances than ammonia (NaOH,

$Ba(OH)_2$ and arginine) gave much broader boundaries, while the weaker bases (creatine, creatinine, glycine) gave values for λ_{90} rather lower than those given by ammonia. The volume-concentration curves for the stronger bases were very sharp at the point of initial break-through, but became very flat as the concentration of the effluent reached 50–60 % of that of the inflowing solution. This effect is clearly illustrated by Fig. 7, in which the volume-concentration curves have been plotted for a series of buffer

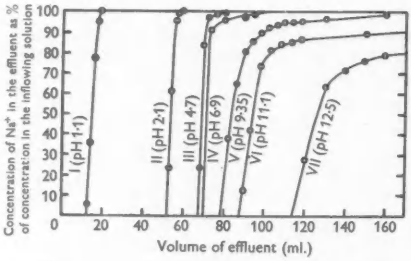
Concentration of Na+ in the effluent as % of concentration in the inflowing solution
Volume of effluent (ml.)
I (pH 1·1)
II (pH 2·1)
III (pH 4·7)
IV (pH 6·9)
V (pH 9·35)
VI (pH 11·1)
VII (pH 12·5)

Fig. 7. Volume-concentration curves showing the shape of the fronts obtained on 'Zeo-Karb 215' (80–100 mesh) with buffer solutions containing Na^+ (0·053 M) and various anions. I, NaCl + HCl (pH 1·1); II, NaCl + HCl (pH 2·1); III, Na acetate + acetic acid (pH 4·7); IV, NaH_2PO_4 + NaOH (pH 6·9); V, boric acid + NaOH (pH 9·35); VI, boric acid + NaOH (pH 11·1); VII, NaOH (pH 12·5).

solutions in which the concentration of the Na ion was maintained at 0·053 M and the pH adjusted by addition of various anions. The experiments were carried out on 'Zeo-Karb 215' (1·44 g. dry wt., 80–100 mesh) packed into filtration tubes 9 mm. in diameter to form a column 7·5 cm. high. At low values of the pH the fronts were in every way typical of those given by weak bases, but under strongly alkaline conditions the fronts became very diffuse, although they remained sharp at the point of break-through and showed no tendency towards a sigmoid shape.

The diffuse character of the boundaries at high values of pH is believed to be due to the reaction of strong bases with the free phenolic-hydroxyl groups in the resin, and fronts of the shape found would be expected if this reaction were slower than the reaction of the bases with $—SO_3H$. The poor boundaries given by the stronger bases, together with the excessive swelling of the resin that occurs in strongly alkaline solutions, renders 'Zeo-Karb 215' rather unsuitable for use with bases stronger than ammonia. This is unfortunate, since the important amino-acid arginine is included in this category.

The relation of λ_{90} to the yield of pure component obtainable from a separation. Inspection of Fig. 8 shows that the approximate percentage yield of pure material recoverable from the band of the first component (A) issuing from the column is given by

$$\text{percentage yield} = \left(1 - \frac{\lambda_{90}^b}{2l_e^a}\right) \times 100, \qquad \text{(xi)}$$

and that of any other component (M) is given by

$$\text{percentage yield} = \left(1 - \frac{\lambda_{90}^m + \lambda_{90}^n}{2l_e^m}\right) \times 100. \qquad \text{(xii)}$$

(Symbols are as indicated in Fig. 8.)

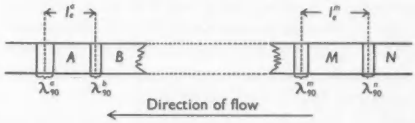

Fig. 8. Diagram of the bands (A, B, ..., M, N) on a displacement chromatogram showing the boundaries of the bands (boundary width, λ_{90}^a, λ_{90}^b, ..., λ_{90}^m, λ_{90}^n).

In some cases (where the values for λ_{90} are small, and the components in the system are well separated in strength as bases) it may be sufficient to make the simplifying assumption that all the values for λ_{90} are equal, in which case the percentage yield of the first component becomes

$$\left(1 - \frac{\lambda_{90}}{2l_e^a}\right) \times 100, \qquad \text{(xiii)}$$

and that of any other component (M) is

$$\left(1 - \frac{\lambda_{90}}{l_e^m}\right) \times 100. \qquad \text{(xiv)}$$

The degree to which this procedure is valid is indicated by the data in Table 2, which lists values for λ_{90} for a number of boundaries on 'Zeo-Karb 215'.

Table 2. *The width (λ_{90}) of various boundaries on 'Zeo-Karb 215'*

Boundary*	Mesh/in.	Rate (S) (cm./hr.)	λ_{90} (cm.)
H^+-ammonia	40–60	10	2·00
H^+-ammonia	80–100	10	1·25
H^+-lysine	80–100	5	2·40
H^+-histidine	80–100	5	2·56
H^+-glycine	80–100	5	0·56
H^+-serine	80–100	5	1·27
H^+-glutamic acid	80–100	5	2·10
H^+-aspartic acid	80–100	5	2·10
Histidine-ammonia	80–100	5	2·14
Glycine-histidine	40–60	10	3·10
Creatinine-ammonia	80–100	5	0·80
Glycine-creatinine	80–100	5	0·80
Aspartic-glycine	40–60	10	3·50

* 'H^+-ammonia' indicates the boundary of a front due to ammonia progressing down a column of the cation exchanger in its acid form, at a rate of S cm./hr.; 'histidine-ammonia' indicates the boundary of a front due to ammonia when this is displacing histidine from the cation exchanger.

The boundaries in which a base displaces H^+ are generally rather narrower than those in which one base is displaced by another. However on the 80–100 mesh resin the values lie in the range 0·56–2·56, while on 40–60 mesh resin the corresponding range is 2·00–3·50.

For the purpose of calculating the minimum length of column required to carry out a specified separation, and to form an estimate of the expected yield, we may adopt a tentative average value for λ_{90}. If this value is taken as $\lambda_{90} = 3{\cdot}0$ for separations on 40–60 mesh resin at $S = 10$ cm./hr., the 'expected yields' for the separation shown in Fig. 4 may be calculated as follows:

The column contains 0·7 g. (dry wt.) of 'Zeo-Karb 215'/cm. length. Since 0·10 N-NH_3 solution was used for displacement the data in Fig. 3 shows that, at equilibrium, ϵ (histidine) = 2·82 mmol./g., ϵ (glycine) = 2·4 mmol./g. and ϵ (aspartic acid) = 1·98 mmol./g. The heights of column occupied by histidine, glycine and aspartic acid are, therefore, 7·2, 15·9 and 12·8 cm. respectively. Thus the 'expected yields' of aspartic acid and histidine (from (xiii)) are 88·4 and 79·2 % respectively, while the expected yield of glycine (from (xiv)) is 81·2 %. These figures compare with the values found: aspartic acid, 81·5 %; histidine, 80·5 % and glycine, 77 %.

DISCUSSION

The application of the principle of displacement chromatography to columns packed with cation-exchange resins enables a complicated mixture of bases and ampholytes to be separated into a series of cuts, each of which contains those components of the mixture which have closely similar strength as bases. Thus a mixture of amino-acids may be resolved into a series of fractions each containing ampholytes of similar isoelectric point. The isolation of pure components (from a single passage through the column) may only be expected when the initial mixture does not contain bases of closely similar strength, and in general, for ampholytes, a difference in isoelectric points of 0·5–1·0 pH unit is required to effect a useful separation between any two components.

The present communication is confined to experiments with individual bases and amino-acids and to the separation of simple mixtures, the purpose of the work being to establish the principles of the method, to collect adsorption data and to acquire a detailed picture of the relevant properties of a typical commercial resin of proven suitability to the purpose. A simple theoretical treatment has been outlined, but the physics of the rather complicated processes involved has not been pursued beyond the point required to develop a working rationale.

It is inherent in the nature of the displacement chromatogram that single-component bands overlap with one another over a narrow zone which has been termed the 'boundary of the band'. The width of the boundary determines the fraction of the component which may be isolated in a pure condition, and is affected by the experimental arrangement adopted and by the properties of the resin itself. Two properties of the resin are therefore of overriding importance, the adsorptive properties under different conditions of hydrogen-ion concentration and the width of the boundaries observed under standard experimental conditions.

These two factors have been studied in some detail and the results obtained provide the data required for the rational planning of attempts to fractionate complicated mixtures of biological origin on a fairly large scale.

SUMMARY

1. The principle of displacement chromatography has been applied to the separation of bases and ampholytes on columns packed with the synthetic cation-exchange resin 'Zeo-Karb 215'.

2. Separations were followed by observing changes in the composition of the effluent solutions. Four general methods were adopted for this purpose: (*a*) continuous measurement of electrical conductivity; (*b*) continuous measurement of pH; (*c*) titration of successive small fractions; (*d*) qualitative analysis of successive fractions by means of the filter-paper chromatogram.

3. Data for the adsorption of various bases and amino-acids on 'Zeo-Karb 215' are given in the form of retention isotherms. The curves are satisfied by the well-known equation of Langmuir, provided the change in pH with dilution of the solute is small.

4. Effective separation between two components of a mixture of ampholytes was obtained on the column, provided that the components were separated by more than 0·5–1·0 pH unit in isoelectric point.

5. The effect of hydrogen-ion concentration on the adsorption of bases by 'Zeo-Karb 215' was investigated; over the range pH 1–7 the sulphonic acid residues in the resin constituted the only reacting group. Under more alkaline conditions, phenolic residues also react with bases.

6. The reaction of phenolic residues with strong bases reaches equilibrium slowly, and for this reason resins that contain residual phenolic groups are unsuitable for the separation of bases stronger than ammonia.

7. The effect of the particle size of the resin on the width of the boundaries of single-component bands was investigated. Fine grinding was found to be essential for the production of sharp bands, and for general use a resin graded between 40 and 60 mesh/in. wire sieves was satisfactory.

8. An increase in the rate of progression of the bands down the column decreases the sharpness of the bands. On 'Zeo-Karb 215' (40–60 mesh/in.) a rate of progression of 10–15 cm./hr. gave satisfactory results.

9. Equations have been derived permitting the calculation of the proportion of the column occupied by a component, the width of the boundaries and the expected yield of pure components in separation experiments.

10. Details of a specimen separation are given, and the yields obtained compared with estimates derived from theory.

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Purification of Alkaline Phosphatase

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Thoai, Roche & Sartori (1944) claimed to have isolated and crystallized the alkaline phosphatase (phosphomonoesterase) of ox kidney. The isolation of similar crystals from dog intestinal mucosa by the same workers has been reported by Roche (1946). These authors pointed out that the crystals lost their activity after a few recrystallizations. This they attributed to a denaturation or loss of coenzyme. By dialysis and reactivation Thoai, Roche & Roger (1947) obtained amorphous preparations which were more active than the crystals. The procedure adopted for the preparation and purification of the enzyme was a modified Albers & Albers (1935) technique.

Following the same procedure, we have obtained very similar crystals which, in the first instance, showed high phosphomonoesterase activity. But after careful purification of these crystals it has been found that they are principally composed of inorganic matter which appears to be magnesium orthophosphate. After careful dialysis for a prolonged period the crystals can no longer be obtained from the dialyzed enzyme preparations by any procedure tried; but the enzyme activity is not affected and can be completely restored by adding magnesium to the buffer substrate mixture.

We have also tried other effective procedures for the further purification of phosphatase, e.g. tryptic digestion followed by prolonged dialysis (cf. Schmidt & Thannhauser, 1943; Abul-Fadl & King, 1949). The products thus obtained show much higher activity than the mixtures containing the crystals; but several attempts to crystallize such protein material, of very high phosphatase activity, have been unsuccessful.

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EXPERIMENTAL

Preparation of phosphatase extracts and purification by acetone

Fresh healthy ox kidneys are decapsulated, freed from fat, well washed and twice minced in a machine. They are then thoroughly mixed with an equal weight of 25% aqueous acetone containing 10% (v/v) of toluene and ethyl acetate (1:1). Autolysis is allowed to proceed at room temperature for 2–3 days with occasional thorough shaking. The mixture is strained through muslin, filtered clear, and then treated in the cold with acetone to a concentration of 55%. The precipitate obtained is separated by centrifuging, washed twice with pure acetone and dried *in vacuo*. A 2% aqueous filtered solution of the precipitate is then subjected to fractional precipitation with cold redistilled acetone at 0°. The fraction obtained at an acetone concentration of 38–50% is mainly protein in nature, and possesses considerable phosphatase activity. When left for 24–36 hr. in the cold room (at 5°) it sets into a crystalline mass: these crystals were claimed by Thoai *et al.* (1947) to be the crystalline enzyme.

Dog intestines from normal animals have also been used for similar preparations. After thorough rinsing with saline solution, the gut is cut open longitudinally, and the mucosa is scraped off with the aid of wooden spatulas. Autolysates of intestinal mucosa, prepared as described for kidneys, have yielded highly active enzyme concentrates from which similar crystals have also been obtained.

Purification by tryptic digestion and dialysis

The fractions obtained between 38–50% acetone concentration were treated with a 0·2% solution of a highly active purified trypsin obtained from Armour Ltd.

The pH was adjusted to 8·0 with Na_2CO_3 solution and the mixture incubated at 37° for 3–4 hr. The preparation was then shaken with 5% kaolin and centrifuged or filtered. This procedure was repeated twice to remove any remaining trypsin. The phosphatase activity was not affected. The enzyme was now precipitated in 0·9 saturated $(NH_4)_2SO_4$ solution. The precipitate was collected by centrifuging, and dialyzed in collodion bags at room temperature for 3–4 days, against a large volume of distilled water frequently changed. The activity and total N were determined each day, and dialysis was stopped when the activity/mg. total N remained constant for two successive estimations.

Activity determination

Activity has been determined in two ways: (*a*) in Roche P units, by estimation of orthophosphate liberated from 0·02 M-β-glycerophosphate in veronal buffer at pH 9 at 37°; (*b*) in King phenol units, by estimation of phenol liberated from 0·005 M-sodium phenylphosphate in Na_2CO_3-$NaHCO_3$ buffer at pH 10 at 37°. Determination of the activity by the two methods allows of comparison with former results published both by ourselves and by other authors.

Magnesium was added to give a concentration of 0·01 M. Phosphorus was determined by Briggs's (1922) method or those of King (1932) and Alleu (1940), phenol by the Folin-Ciocalteu method (for details see King, 1947), and total N by the micro-Kjeldahl procedure.

(*a*) *Determination of activity in terms of Roche phosphate units.* In a test tube marked at 10 ml. are placed 4 ml. veronal buffer (pH 9), together with 2 ml. 0·1 M-sodium glycerophosphate and 1 ml. 0·1 M-magnesium acetate. The volume is adjusted to 9·9 ml. with distilled water, and the tube allowed to stand in a 37° water bath for 5 min. The highly active enzyme solution (0·1 ml.) is added and hydrolysis is allowed to proceed for exactly 5 min. Trichloroacetic acid (25%, 1 ml.) is added to stop enzyme action and to precipitate any proteins. (Generally the highly active preparations contain negligible amounts of precipitable proteins.) The solution is filtered if necessary, and the inorganic phosphate determination carried out. Controls without any enzyme solution and blanks with enzyme added after acidification must be run parallel with the tests. If the amount of inorganic phosphate exceeds 0·3 mg. P in the whole tube (corresponding to about 5% hydrolysis) the determination is repeated with a conveniently diluted enzyme solution. The Roche unit of phosphatase is defined as the amount of enzyme which will liberate 1 μg. P/min.

(*b*) *Activity by King phenol units.* The Na_2CO_3-$NaHCO_3$ buffer of pH 10 (2 ml., Delory & King, 1945), 0·02 M-disodium phenylphosphate solution (1 ml.) and 0·04 M-magnesium acetate (1 ml.) are mixed in a test tube and warmed to 37°. After addition of 0·2 ml. of the enzyme solution suitably diluted, and incubation of the mixture for 15 min. at 37°, 1·8 ml. dilute (1 in 3) Folin-Ciocalteu reagent are added. The solution is mixed, centrifuged if necessary, and phenol determined in 4 ml. of the clear supernatant fluid (cf. King, 1947). Controls and blanks are also used. The unit of phosphatase is defined as the amount of enzyme which will liberate 1 mg. phenol/15 min.

RESULTS AND DISCUSSION

Dog intestinal phosphatase. Table 1 shows the activity of dog intestinal preparations at different stages of purification. The activity of the mucosa autolysates has been found to vary considerably from one preparation to another. It is important to work with intestines obtained from healthy, well-fed animals, showing no sign of abnormality. Preparations from ill or starving dogs yield solutions of low activity. The results shown in Table 1 are those normally obtained from satisfactory preparations.

Table 1. *Phosphatase activity of dog intestinal preparations during different stages of purification*

Preparation	Activity: Phosphate (Roche units/mg. total N)	Activity: Phenol (King units/mg. total N)
1. Autolysis for 3 days in 25% acetone. (Autolysate filtered clear, light yellow)	650–800	70–85
2. Crude Albers, precipitated by 55% acetone from above autolysate	2000–2400	240–300
3. Fractional precipitation by acetone 38–50%, 0–4°	5000–8000	600–890
4. Tryptic digestion followed by prolonged dialysis	9360–15700	1172–1600

The amount of mucosa obtained from the whole small intestine from a normal dog is generally about 100 g. which is left to autolyze as described above. The clear filtered autolysate is generally light straw-yellow in colour and has a total nitrogen content of about 0·7–1·6 mg./ml. The crude Albers preparation obtained by precipitating with 55% acetone in the cold has a higher activity (units/mg. total nitrogen) than the autolysate, but its comparatively low total nitrogen content (7–9%) indicates contamination with non-nitrogenous materials (possibly carbohydrates) as well as inorganic matter.

On fractional precipitation with cold pure redistilled acetone the fractions obtained below 38% acetone concentrations are relatively poor in enzyme activity. The fractions obtained with higher acetone concentrations (38–50%), on the other hand, are rich in enzyme activity. By carrying out this stage carefully in a cold room at approximately 0°, adding the chilled pure acetone gradually with constant stirring and separating every fraction by thorough

centrifuging in the cold room, it is possible to obtain highly active products, containing about 12–14% nitrogen.

This fraction, however, is always contaminated with inorganic matter, consisting mainly of magnesium phosphate which crystallizes when the preparation is kept in the cold. The residual acetone evaporates slowly, and the inorganic crystals are gradually formed in the viscous protein solution, which adheres intimately and takes the shape of the crystals, thus giving the impression that the enzyme itself has crystallized.

The enzyme preparation at this stage could not be further purified by fractional precipitation with acetone. We have tried several ways of purification involving fractional salting out with ammonium sulphate, adsorption with alumina and kaolin, simple dialysis and electrodialysis, but none has given satisfactory results.

Tryptic digestion under the described conditions, however, followed by treatment with kaolin, salting out with ammonium sulphate and then prolonged dialysis, has been found very effective in achieving further purification. The relative stability of the intestinal phosphatase towards dialysis has been an additional favourable factor in effecting purification of this enzyme after tryptic digestion in this way. The preparation obtained after dialysis is an almost colourless, highly active, aqueous solution. If dialysis is very prolonged, the preparation begins to lose its activity with further decrease in total nitrogen. The activity, however, can be completely restored, as described by Thoai *et al.* (1947), by incubating the partially, but not completely, inhibited enzyme preparation with α-amino acids in a slightly alkaline medium (pH $\nearrow$ 9). The best preparation contained 0·14 mg. nitrogen/ml., and had an activity of 15,700 Roche phosphate units or 1600 King phenol units/mg. total nitrogen.

The enzyme was obtained in the dry form without loss of activity by adding pure redistilled acetone to this solution in the cold to a concentration of 80–90%. It separated as a light coagulated precipitate, which settled overnight in the cold, and was separated by decantation and centrifuging. Various attempts to crystallize this preparation, e.g. by allowing to stand in the cold in concentrated solution with and without spontaneous evaporation in a vacuum desiccator, by careful addition of ethanol and acetone, by allowing to stand at 0° in different concentrations of acetone, by additions of various cations or by addition of ammonium sulphate and other salting-out materials like sodium phosphate and magnesium sulphate, were all unsuccessful.

The total nitrogen content of the dry powders obtained from different preparations was 10–11% nitrogen. No carbohydrate residue was detected in any of our final preparations. After prolonged hydrolysis with acid, no reduction of Benedict or Fehling solutions could be detected (cf. Schmidt & Thannhauser, 1943).

Ox-kidney phosphatase. Table 2 shows the activity of ox-kidney preparations during different stages of purification. Our experience with ox kidneys both in England and in France was entirely unsatisfactory. Preparations much weaker in activity than those from dog intestines have always been obtained. The autolysates, as well as the other preparations, are characterized by comparatively low activity and high total nitrogen (generally about 13–15 mg. nitrogen/ml.).

Table 2. *Activity of ox-kidney preparations during stages of purification*

Preparation	Activity: Phosphate (Roche units/mg. total N)	Activity: Phenol (King units/mg. total N)
1. Autolysis at room temperature for 3 days in 25% acetone in presence of 0·1 vol. toluene-ethylacetate. Autolysate filtered clear, straw yellow	100–135	10–14
2. Crude Albers precipitated by 55% acetone from above autolysate	32–600	35–65
3. Fractional precipitation by acetone 38–50%, 0–4°	840–1200	90–130
4. Tryptic digestion and dialysis	2000–3500	250–400

The 38–50% acetone fraction obtained from crude Albers's preparations contains about 14% total nitrogen, and is relatively poor in activity. It has a considerable yield of crystals (much more than the corresponding intestinal preparation with higher activity), presumably because of the high phosphate content of this organ. Moreover, the relative instability of the kidney phosphatase during dialysis makes efficient purification of the enzyme by this procedure rather difficult. In this respect it differs from the intestinal enzyme which is more stable; also it is not reactivated to the same extent as the latter by amino-acids after partial inactivation through dialysis. This will be discussed in detail elsewhere. Tryptic digestion effected considerable purification of this enzyme.

In Table 3 are given some analytical data of two batches of crystals obtained from two kidney preparations. These were obtained from the first crystallization after thorough washing with cold water and rapid centrifuging. These crystals are soluble with difficulty in water, but are more soluble in dilute alkali or acid. They contained 12–15% phosphorus, all of which was present as orthophosphate, 4–7% carbon and very little nitrogen. The fact that the carbon and nitrogen contents are so small and so variable suggests that these are due to organic

impurities and are not chemical constituents of the crystals. The ash consisted entirely of magnesium pyrophosphate. Magnesium was determined microgravimetrically as oxinate by precipitation with 8-hydroxyquinoline (Berg, 1935), and phosphorus determined by the King (1932) method, after hydrolyzing the pyrophosphate into orthophosphate. The figures shown in the table are very close to those of $Mg_2P_2O_7$. Microtests for calcium and zinc were negative. No ammonia could be detected in the crystals. Comparison with two crystalline forms of magnesium orthophosphate, as shown in Table 3, shows the close similarity of these crystals with either one or the other, with only slight differences due to the presence of traces of organic impurities which are very difficult to remove, and also of water of crystallization which can easily vary when crystals are kept at room temperature. It is concluded that these crystals are magnesium orthophosphate. It appears, therefore, that the alkaline phosphatase has not been crystallized, but is easily adsorbed by magnesium orthophosphate, and, possibly, by other sparingly soluble mineral salts.

Table 3. *Analytical data for crystals obtained from kidney phosphatase*

	Composition			
	Magnesium phosphate		Crystals from phosphatase	
	Theory for $MgHPO_4.3H_2O$	Theory for $MgHPO_4.7H_2O$	Batch 1	Batch 2
C*	—	—	7·05	4·3
H*	4·02	3·02	2·36	2·04
N*	—	—	2·24	Trace
P	17·7	12·6	12·5	14·7
Mg	13·9	9·86	10·0	11·27
Ash (as $Mg_2P_2O_7$)	62·1	45·2	45·8	60·2
P/Mg	1·27	1·27	1·25	1·30
	Theory for $Mg_2P_2O_7$		Ash of crystals	
P	28·0		26·5	27·8
Mg	21·6		21·7	22·5

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SUMMARY

1. The purification of alkaline phosphatase from dog intestinal mucosa and ox kidney has been studied by different procedures, and highly active preparations were obtained from mucosa.

2. The crystals which were claimed to be pure alkaline phosphatase have been identified as magnesium orthophosphate, with adsorbed phosphatase.

3. Attempts to crystallize the highly active intestinal phosphatase were unsuccessful.

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Purification of Faecal Alkaline Phosphatase

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The presence of considerable amounts of alkaline phosphatase in dog faeces was first reported by Armstrong, King & Harris (1934). Since then, several attempts have been made to separate and purify this enzyme. Armstrong (1935) described a method by which a very potent phosphatase-containing powder could be obtained from dog faeces within 48 hr. King & Delory (1939) repeated this preparation, but did not succeed in raising its potency above that achieved by Armstrong. Chen, Freeman & Ivy (1940) concentrated the faecal phosphatase by adsorption on kaolin in an acid medium and elution by alkaline buffer. Their enzyme preparations, however, were less active than those described by Armstrong.

The present work was undertaken for the purpose of obtaining the faecal phosphatase in the purest condition possible, and to attempt its crystallization.

By the use of several methods of purification, it has been possible to obtain preparations with higher activities than those obtained by Armstrong (1935). Several attempts at crystallization were unsuccessful. With the procedure outlined by Armstrong, we encountered many difficulties, and the preparations obtained were, in the majority of cases, much less active than his. Methods of purification have been studied, and an improved procedure is described.

EXPERIMENTAL

The main features of the method adopted were those originally described by Armstrong (1935). The following precautions, modifications and additions, some of them taken from the procedures used for kidney and intestinal phosphatase by Abul-Fadl, King, Roche & Thoai (1949) have been found useful. (1) Avoidance of any change of the pH towards the acid side, as the faecal phosphatase is very sensitive to acid, even at 0°. (2) Fractionation of the aqueous extract with $(NH_4)_2SO_4$. (3) Decolorization with minimum loss of activity. (4) Removal of inactive protein material from the final enzyme concentrates by short tryptic digestion followed by careful electrodialysis. The whole procedure is outlined below.

Estimation of activity was in terms of King phenol units as described by Abul-Fadl *et al.* (1949).

A. *Preparation of a clear extract from the faeces*

About 1 kg. of dog faeces, collected within 24 hr. of being passed, is placed in a beaker together with cracked ice, and tap water added to make the total volume about 2 l. The contents are stirred thoroughly till uniform and then strained through a wire sieve. The sludge is tested by litmus paper and made alkaline with ammonia. It is then poured into a fluted filter paper and left in the ice box to filter overnight. The filtrate, if not absolutely clear, should be treated with 5% kieselguhr or kaolin and refiltered with suction.

The dark brown filtrate, which should be free from any turbidity, shows an activity of 100–150 King phenol units/ml. and contains about 1·2–0·6 mg. protein N/ml. With a few drops of toluene added, the solution retains its activity for several days at 0°. To assure a high degree of activity, faeces should be collected from healthy, well-fed dogs, discarding any samples which have become dry.

It will be noted that preliminary autolysis, as in the case of kidney and intestine, is here unnecessary since the faecal phosphatase is readily obtained in a soluble form.

B. *Precipitation with ammonium sulphate*

Preliminary experiments showed that precipitation with acetone at this stage, as was used in the case of kidney and intestinal preparations, gives preparations of poor activity whilst $MgSO_4$, even in full saturation, was of little use in salting out the enzyme. Half saturation with $(NH_4)_2SO_4$, however, precipitates about 90% of the phosphatase, while with 0·8 saturation precipitation is almost complete. To each 100 ml. clear filtrate, cooled to 0°, 40 g. $(NH_4)_2SO_4$ (A.R.) are added with stirring. On standing for 10–15 min. in the cold a precipitate is formed which rises to the surface. Most of the clear underlying liquid can be removed by siphoning, and the precipitate readily collected and separated by rapid centrifuging.

The use of acetone at this stage to help salting out, as described by Armstrong (1935), leads to much loss of activity in the aqueous acetone solution. The loss in some experiments amounted to about 40%.

The precipitate thus obtained gives a dark brown turbid solution. The activity is about 600–750 units/ml., with a high protein N amounting to as much as 6·5 mg. N/ml. in some experiments.

C. *Decolorization by activated charcoal*

This is the most critical step in the purification of the faecal phosphatase. Much enzyme activity may be lost if the steps are not carefully controlled. The difficulties encountered are due to the following: (1) The faecal enzyme is unstable in slightly acid medium even in the cold, but for effective decolorization the medium must be slightly acid. (2) Adsorption of the enzyme on the activated charcoal cannot be reversed.

Glycerophosphate buffer adjusted with acetic acid to pH 5·2–5·4 and containing 0·5 M-$MgSO_4$ is the best medium for effective decolorization with removal of inactive protein material and minimum loss of enzyme activity. β-Glycerophosphate appears to protect the enzyme against inactivation due to changes in pH, while Mg increases the enzyme affinity for substrate, thus minimizing the risk of adsorption on the charcoal.

The precipitate obtained from B is dissolved in 150 ml. 0·02 M-sodium β-glycerophosphate solution previously adjusted with dilute acetic acid to pH 5·2–5·4, and 15 g. crystalline $MgSO_4.7H_2O$ dissolved in the solution. The procedure is conducted in the cold at about 0°. Activated charcoal (1·5 g.) is now added and the mixture is well stirred for 10 min. It is then filtered through a folded filter paper previously prepared by passing through it 1% charcoal suspension in water. The filtrate is almost colourless, or only slightly coloured, and shows an activity of 570–700 units/ml.

The solution is transferred into collodion or cellophan bags of 50 ml. capacity and left to dialyze against distilled water overnight to eliminate most of the Mg, in the presence of which inactivation is likely to occur, and has been observed in many cases. The dialyzed solution, increased in volume by osmosis, is now subjected to further purification as it still contains a lot of inactive protein.

D. *Digestion with trypsin*

The effect of tryptic digestion on purification of phosphatase has been discussed by Abul-Fadl & King (1949). A potent commercial trypsin (Armour Ltd.) is thoroughly mixed with the enzyme solution in a concentration of 0·2%. The pH is adjusted to 8·0 with Na_2CO_3 and the mixture left to stand in a thermostat at 37° for 3–4 hr. At the end of this time 5% kaolin is added, stirred for 10 min. and then separated by centrifuging. This procedure is twice repeated in order to eliminate any residual trypsin. If any colour is still present, it can be removed at this stage by a second charcoal treatment (1%), which will also help in removing traces of trypsin, with practically no loss in enzyme activity. The medium need not be acidified in this operation since the colour is only slight and complete decolorization can be easily effected.

The activity now ranges between 400 and 500 units/ml. and the protein N from 0·2 to 0·3 mg. N/ml. Thus a high degree of purification has been achieved at this stage; but the preparation is still rich in non-protein N, which can be eliminated by prolonged dialysis in collodion or cellophan bags against running distilled water.

E. *Further purification by electrodialysis*

Kutscher & Wolbergs (1935) claimed to have obtained the prostatic phosphatase in a high state of activity by long electrodialysis. On the other hand, Albers & Albers (1935) reported a continuous loss of activity in kidney-phosphatase preparations subjected to electrodialysis.

We have studied the behaviour of kidney, intestine and faecal phosphatase towards electrodialysis and the detailed results will be published later. With the faecal phosphatase preparations we have been able to attain a further degree of purification by virtue of the denaturation of the remaining proteins which occurs during the electrodialysis.

The apparatus described by King & Dolan (1934) has been used. The clear faecal phosphatase solution is placed in a 150 ml. capacity cell, and distilled water is run continuously in the two parchment thimbles containing the electrodes. The current is kept low (10–20 ma.) throughout the experiment which lasts usually for 24–30 hr. Care must be taken to keep water running in the thimbles and to stir the dialyzed solution continuously. If a current of N_2 is used for stirring, it must be slow, so as not to form excessive froth which causes surface inactivation of the enzyme. Drops of chloroform are added as a preservative and to minimize frothing.

No physical change is observed in the enzyme solution during dialysis. The activity and protein N, which are tested regularly, show no significant variation throughout the experiment. The enzyme solution remains clear, and only starts to show turbidity near the end of the experiment when diminution in activity is also noticed. On the other hand, turbidity is always found inside the cathode chamber, owing to elimination of nitrogenous and other impurities. If the dialysis is unduly prolonged or the water circulation is stopped, the enzyme solution starts to show turbidity, and precipitation may occur with complete loss of activity.

F. *Precipitation with acetone*

At the end of electrodialysis there is no change of activity in relation to the protein N, and the solution is perfectly clear. The enzyme solution is now cooled to 0° and pure cold acetone is added to a concentration of about 60%. A flocculent precipitate collects at the bottom on leaving to stand in the cold. This can be separated by pouring off the supernatant fluid, centrifuging and washing with pure acetone.

The precipitate thus obtained, however, is not now completely soluble in water. It seems that part of the proteins has been denatured. Distilled water (20 ml.) is now thoroughly stirred with the acetone precipitate, allowed to stand in the cold for about 30 min., and any insoluble part spun down. The clear supernatant fluid is reprecipitated with 60–80% acetone in the cold, separated and washed with acetone by centrifugation. It is finally dried over $CaCl_2$ or soda lime in a vacuum desiccator.

The precipitate dries in thin, almost transparent scales. It can be pulverized into a white or buff-coloured powder showing an activity of 1166–1300 units/mg. N. The total N is 10–11% and protein N is 5·6–6·2%. The yield is 0·2–0·4 g./kg. faeces.

DISCUSSION

The activity/mg. dry powder is of the order of 95–120 units which corresponds to 190–240 King-Armstrong units. This activity is higher than that recorded by Armstrong (1935), whose best preparation was of about 185 King-Armstrong units.

These purified faecal phosphatase preparations are similar to the intestinal preparations described by Abul-Fadl *et al.* (1949) in their nitrogen content and in the absence of any detectable carbohydrate residue. On the other hand, there is a tendency to get more active enzyme preparations from the mucosa since our best faecal preparation shows an activity of 1300 units/mg. nitrogen as compared with that from mucosa which was about 1600 units/mg. nitrogen. In general, it has been noticed that the most active purified preparations were always obtained from starting materials which were originally of a very high order of activity. This suggests that the enzyme may be originally present in the tissues in quite different amounts or degrees of activity.

As in the case of intestinal phosphatase, repeated attempts at crystallization of the purified faecal enzyme have been unsuccessful.

SUMMARY

1. A method for the purification of faecal alkaline phosphatase is described.

2. Amorphous phosphatase preparations of very high activity have been prepared, but all attempts to crystallize them have failed.

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Purification of Alkaline Phosphatase by Tryptic Digestion

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After fractional precipitation of kidney, intestinal and faecal phosphatase with acetone in the cold, further attempts at purification by either repeated acetone or ammonium sulphate fractionation were ineffective. Treatment with aluminium hydroxide was likewise of little use, and there was considerable loss of enzymic activity. Digestion with trypsin, at this stage, proved to be of great advantage in eliminating inactive proteins which are otherwise difficult to remove.

In most of the current procedures for the preparation of phosphatase, autolysis is used as an indispensable stage in the liberation of the enzyme from the tissues. Ehrenward (1933) discovered that the activity of alkaline phosphatase is not affected by trypsin. Kutscher & Pany (1938) found later that acid phosphatase is resistant to papain. Schmidt & Thannhauser (1943) used artificial proteolysis in the form of tryptic digestion for bringing the calf intestinal phosphatase into solution. Fischer & Greep (1948) have recently reported on an 'activation' of the alkaline phosphatase by trypsin. Thus the tryptic digestion of crude phosphatase preparations is well known, but its effect on highly concentrated purified enzyme preparations has not been well investigated. For this reason, it seemed desirable to conduct such a study to find out the best conditions for purification with minimum loss of enzyme activity.

EXPERIMENTAL

Enzyme solutions from ox kidney and dog intestinal mucosa treated by fractionation with acetone in the cold, and dog faecal phosphatase prepared by fractional precipitation with $(NH_4)_2SO_4$ and charcoal decolorization, have been used. A very potent trypsin powder (U.S.P.X., Armour Laboratories, Chicago) has been used throughout. The enzyme solutions were adjusted to pH 8 by Na_2CO_3 solution and kept at 37° with various concentrations of trypsin powder. Activities expressed in King phenol units in relation to protein N by micro-Kjeldahl (see Abul-Fadl, King, Roche & Thoai, 1949) have been determined in the presence of Mg^{++} on samples withdrawn from the incubated enzyme solutions at regular intervals.

After tryptic digestion there is always a considerable amount of non-protein N, which is best eliminated by prolonged dialysis.

RESULTS AND DISCUSSION

Table 1 shows the effect of tryptic digestion on faecal and intestinal phosphatases. An initial increase of the enzyme activity in relation to protein nitrogen during the first few hours of incubation is followed by a gradual fall. It is, therefore, inadvisable, with 0·1–0·2% trypsin, to incubate for longer than 2–3 hr. at 37°. This depends on the degree of contamination of the enzyme with inactive protein. With kidney preparations (Fig. 1), however, a longer

Table 1. *Effect of trypsin digestion on partially purified alkaline phosphatase*

Enzyme solution	Trypsin concentration (%)	Time of incubation at 37° (hr.)	Protein N (mg./ml.)	Phosphatase activity (Units/ml.)	Phosphatase activity (Units/mg. protein N)
Intestinal	0·1	0	0·20	300	1500
		2	0·18	300	1720
		4	0·16	255	1569
		6	0·16	255	1569
		8	0·16	240	1480
Intestinal	0·2	0	0·17	315	1852
		1·5	0·14	293	2192
		3	0·13	243	1868
		4·5	0·12	225	1800
		6	0·12	200	1660
Faecal	0·5	0	0·40	240	600
		2	0·28	240	857
		4	0·24	200	833
		6	0·24	185	770
		24	0·24	144	600
		30	0·24	144	600*
		48	0·24	126	525*
		60	0·24	102	425

* More trypsin added.

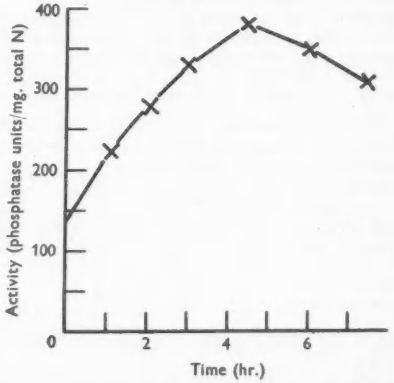

Fig. 1. Effect of tryptic digestion on kidney phosphatase. 0·1% trypsin, 37°, pH 8·0.

incubation is required (4–5 hr.) for the maximum activity relative to protein nitrogen to be attained, since these are more contaminated by inactive protein than either faecal or intestinal preparations. It is interesting to note that trypsin does not appear to act on the protein part of the enzyme, since the protein nitrogen of the incubated enzyme solution remains constant after a certain time, and is not affected by any further incubation, even if more trypsin is added. The decline in enzyme activity appears to be due to inactivation of the enzyme, when incubated in alkaline buffer, rather than to direct trypsin action on the enzyme itself. This inactivation was pointed out by Fischer & Greep (1948) to be much more extensive in the absence of trypsin than when it is present. Thus trypsin, in addition to digesting contaminating proteins, seems to protect the enzyme to a certain extent against inactivation during incubation in alkaline buffer. Excess of trypsin, however, does not seem to increase the degree of protection of the enzyme; on the contrary, it seems to decrease it, or to cause inhibition of the phosphatase.

SUMMARY

1. Further purification of the alkaline phosphatase, beyond that attainable by fractional precipitation and salting-out procedures, was obtained by incubation with 0·1–0·2 % trypsin at 37° for a period of not more than 4 hr.

2. Addition of more trypsin, or incubation for longer periods, resulted in loss of enzyme activity.

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Reactivation of Alkaline Phosphatases after Dialysis

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Albers, Bayer, Bohnenkamp & Muller (1938) ascribed the inactivation of the kidney alkaline phosphatase through prolonged dialysis to a separation of the phosphatase into an inactive protein apoenzyme and a dialyzable coenzyme or prosthetic group. These workers showed that this coenzyme cannot be magnesium. It appeared to be a labile organic substance, which could be obtained by incubating the holoferment at 100° for a few minutes in a neutral medium. This treatment destroyed the apoenzyme while not affecting the coenzyme. By mixing the latter with an enzyme preparation partially inactivated by dialysis or previous incubation in an acid medium, active phosphatase solutions were obtained. On combining the dialyzable liquid with the inactivated protein solution of dialyzed kidney preparations, with the addition of magnesium, Albers (1939) claimed to have obtained an almost 100 % reactivation. von Euler & Hahn (1947) described a partial reactivation of intestinal phosphatase, inactivated by dialysis at pH 4·5, by the addition of boiled enzyme solution. Cloetens (1941*a*, *b*, 1942), after repeating Albers's experiments, came to the conclusion that the activating action of a heated enzyme solution (Kochsaft), or the dialysate liquid, was not due to the presence of a cophosphatase, but more probably to a zinc or cobalt ion, which is present in very small amount and which forms a dissociable complex. After studying the influence of different metallic ions, as well as substances which form weakly dissociable combinations with metals, Cloetens concluded that alkaline phosphatase is composed of a slightly active or completely inactive part, EG_2^1 (i.e. protein + metal-combining groups), and the two metals magnesium and zinc. This postulate was supported by the work of Hove, Elvehjem & Hart (1940), who used zinc salts in combination with amino-acids and with protein split products (from autolyzed mucosal tissue) to activate dialyzed intestinal phosphatase. Thoai, Roche & Roger (1947) reported the inactivation of intestinal phosphatase by prolonged dialysis at 37°, and its complete reactivation by incubation with amino-acids in alkaline medium and determination of the enzyme activity in presence of certain inorganic ions. Comparing these findings with that of Albers, it would appear possible that the dialyzable prosthetic group may be a combination of a metal other than magnesium (possibly zinc) with a compound containing amino-acid groups.

In the present investigation an attempt has been made, using ordinary dialysis as well as electrodialysis, to obtain a clearer conception of the nature of the dialyzable factors indispensable for the activity of alkaline phosphatase. It has been found that magnesium and a nitrogenous non-protein dialyzable substance, which migrates towards the cathode during electrodialysis, are responsible for activating the apoenzyme. No evidence for the identity of this dialyzable substance with simple α-amino-acids, or for the presence of zinc, has been obtained. Differences among alkaline phosphatase preparations from different sources have been found, and the results are discussed.

EXPERIMENTAL

King & Dolan's (1934) electrodialyzing apparatus has been used throughout. It consists of a glass cell of suitable capacity containing two units of electrode chambers immersed in the enzyme solution. Each unit consists of a parchment dialyzing thimble containing the electrode surrounded by distilled water. Because of the shortage of platinum this has been used only for the cathode, while a carbon electrode has been found quite satisfactory as an anode. The two electrode chambers were either connected together, as when water is circulated through them, or left unconnected, according to the requirements of the experiment. A mechanical stirrer was used, since the bubbling of N_2 was found to cause some inactivation due to frothing. A mercury-vapour-lamp transformer was used as the source of direct current. In some cases a 6 V. battery was used. An ammeter was included in the circuit. The current passing through the enzyme solution was as low as 1·5 ma. and was never allowed to exceed 10 ma.

Enzyme solution and determination of activity. The solutions being dialyzed always contained the enzyme in its highest available state of purity, prepared and the activity determined according to the methods already described (Abul-Fadl, King, Roche & Thoai, 1949; Abul-Fadl & King, 1949).

Nitrogen determination. The micro-Kjeldahl method was used, and was carried out on 0·2 ml. of the clear enzyme solution on which the activity had been previously determined. For protein N the proteins were precipitated with molybdic acid. Digestion was carried out with 2 ml. 50% (v/v) H_2SO_4 containing 1% SeO_2, a small electric heater being used.

Microdetection of certain metals (cf. British Drug Houses Ltd. *Book of Organic Reagents for Analytical Use including Spot Tests*). Magnesium was tested for by the *p*-nitrobenzeneazo-α-naphthol test which is sensitive to 2 μg. Mg; calcium by the picrolonic acid test which is sensitive to 0·01 μg. Ca; and zinc by the resorcinol test, sensitive to 2 μg. Zn.

Procedure for dialysis. The enzyme solution was subjected to prolonged electrodialysis against running distilled water, continuously stirring and using an electric current of 1–3 ma. The enzyme activity was determined at regular intervals, with and without the addition of Mg^{++} to the buffer-substrate mixture. As the activity started to decrease, the two electrode chambers were disconnected from each other, and distilled water was very slowly passed through each chamber separately, and collected in separate containers; or if necessary the water circulation was stopped. The current was gradually increased.

The effect of the anode and cathode liquids, as well as certain other substances, was then tried on the enzyme solution which had been partially inactivated by electrodialysis.

RESULTS

Kidney phosphatase. Table 1 shows the changes in enzyme activity in absence and in presence of magnesium, and in total nitrogen and protein nitrogen, taking place during electrodialysis of kidney alkaline

Table 1. *Effect of electrodialysis on the activity of kidney alkaline phosphatase*

(Slow current of running distilled water in the electrode chambers; electric current started with 1 ma. and gradually increased to 10 ma.)

Duration of electrodialysis (hr.)	Enzyme activity (units/ml.)		mg. N/ml.	
	Without Mg	With 0·01 M-Mg	Total	Protein
0	56	84	4·6	1·30
2	56	84	4·5	1·30
4	56	84	4·3	1·30
6	56	84	4·2	1·30
8	53	84	4·1	1·30
10	50	84	4·0	1·30
12	46	84	3·9	1·30
14	40	82	3·8	1·28
16	34	80	3·7	1·27
18	28	53	3·6	1·25
20	20	36	—	—
26	6	9	3·4	1·23
30	3	4	—	—
34	2	2	3·2	1·20
36	0·8	0·8	—	—
40	0·3	0·3	3·0	1·20

phosphatase solution. A progressive loss of activity occurs, and it appears that at least two important factors are lost during the course of the dialysis. The first of these is magnesium, the addition of which restores the original activity of the enzyme solution during the first 16 hr. of electrodialysis, although the time depends on the current strength. After that a sudden drop in activity takes place followed by a comparatively rapid inactivation of the enzyme. This suggests the splitting off of another factor which cannot be identical with magnesium since the latter, even in increased amounts, fails to restore the initial enzyme activity. It is noticed that the more inactivation takes place the more this factor is lost, and the less the response of the enzyme towards magnesium until finally the latter has almost no effect on the enzyme activity.

The total nitrogen falls gradually with electrodialysis. The protein nitrogen remains constant for a time, and then decreases gradually, the solution becoming turbid especially around the cathode chamber. There is a tendency for the protein nitrogen to remain constant again near the end of the dialysis.

Table 2 shows the strong activating power of the clear cathode liquid after filtration from the inactive precipitate. The cathode liquid alone has no activity, but it has considerable activating power when mixed with the apoenzyme, and this is much more pronounced in the presence of magnesium. The three experiments were carried out on the same solution which had been electrodialyzed in three stages. The activation is very marked during the initial stage of electrodialysis, but after prolonged electrodialysis the cathode liquid does not fully restore the initial activity of the enzyme. It seems probable that the apoenzyme is affected by the prolonged dialysis.

Table 2. *Effect of the liquids in the electrode chambers on kidney alkaline phosphatase partially inactivated by electrodialysis*

(These experiments were carried out on a single enzyme solution which was dialyzed in three stages. At the end of each stage the enzyme solution was mixed with the liquids from the electrode chambers collected during the period of dialysis. The water was circulated through the dialyzing thimbles as slowly as possible.)

	Activity (units/ml.)	
	Without Mg	With 0·01 M-Mg
Stage 1 of dialysis:		
Dialyzed enzyme solution (30 hr.) (+water, 1 : 1)	28	53
After 2 hr. further dialysis (+water, 1 : 1)	20	36
Enzyme solution + anode liquid (1 : 1)	20	36
Enzyme solution + cathode liquid (1 : 1)	38	62
Enzyme solution + anode + cathode liquids (1 : 0·5 : 0·5)	38	61
Cathode liquid alone	0	0
Anode liquid alone	0	0
Cathode + anode liquids	0	0
Stage 2 of dialysis:		
Dialyzed enzyme solution (total 35 hr.) (+water, 1 : 1)	8	11
After 1 hr. further dialysis (+water, 1 : 1)	7	9
Enzyme solution + anode liquid (1 : 1)	7	9
Enzyme solution + cathode liquid (1 : 1)	9	15
Stage 3 of dialysis:		
Enzyme solution after prolonged dialysis (+water, 1 : 1)	5	5·5
Further dialysis (+water, 1 : 1)	0	0
Enzyme solution + anode liquid (1 : 1)	0	0
Enzyme solution + cathode liquid (1 : 1)	1·2	5·3

The anode liquid, inactive in itself, has neither activating nor inhibiting action when mixed with the enzyme solution being dialyzed.

Table 3 shows the effect of magnesium, zinc, amino-acids and cathode liquid added to two enzyme solutions of different initial activities which had been partially inactivated by electrodialysis, singly and in different combinations. Zinc in 0·0002–0·0005 M-concentrations and in presence of 0·01 M-magnesium shows a slight activating property, but inhibits in higher concentrations. Its activating effect, however, is very small compared with that of the cathode liquid. A combination of cathode liquid and 0·0002 M-zinc has less activating power than the cathode liquid alone. The amino-acids (glycine and alanine) in different concentrations, even after previous incubation with the inactive enzyme, do not exhibit any activating property; on the contrary, they inhibit, especially in presence of magnesium.

Table 3. *Effect of magnesium and zinc salts and amino-acids compared with that of cathode liquid on the kidney alkaline phosphatase partially inactivated by prolonged electrodialysis*

(Two enzyme solutions A and B of different initial activity were used.)

	A (units/ml.)		B (units/ml.)	
	No Mg	0·01 M-Mg	No Mg	0·01 M-Mg
Original solutions	40	60	13	20
After prolonged dialysis	3·0	3·4	2·7	3·0
Effect of zinc salt:				
0·01 M	0·0	0·5	0·0	0·7
0·001 M	1·6	5·6	0·7	9·6
0·0005 M	2·0	10·5	1·2	11·5
0·0002 M	2·5	10·5	1·5	11·5
0·0001 M	2·7	5·8	2·0	3·5
Incubation with amino-acids:				
Glycine (0·001 M)	0	0	0	0
Glycine (0·0001 M)	1·5	1·0	0·7	0·5
Alanine (0·001 M)	0	0	0	0
Alanine (0·0001 M)	2·0	1·5	1·5	1·2
Enzyme solution mixed with cathode liquid (1 : 1)	28	55	7	18·5
Enzyme solution mixed with cathode liquid, and 0·0002 M-zinc added	6	22·5	2·5	9·4

Table 4 shows that the activating substance present in the cathode liquid is organic in nature as the ash is not active. It is stable to boiling for 10 min. but is destroyed by a slight excess of ammonia.

A cathode liquid with high coenzyme activity contained 3·9 mg. total solids/ml. and yielded 0·60 mg. of ash, mainly alkaline earths. 'Spot' tests for calcium and for zinc in the ash were negative, while a slight positive reaction for magnesium was obtained. The total nitrogen, 0·66 mg./ml., was equivalent to 20 % of the organic matter. This is consistent with the possibility of an amino-acid or peptide structure being present. Protein was apparently absent since there was no detectable precipitate with molybdic acid.

Table 4. *Effect of boiling, ashing and other treatments on the activating property of the cathode liquid on the kidney phosphatase*

	Activity (units/ml.)	
	No Mg	0·01 M-Mg
Kidney phosphatase after 8 hr. electrodialysis	12·6	19
Enzyme solution + cathode liquid	20	60
Enzyme solution + boiled cathode liquid	20	60
Enzyme solution + redissolved ash*	12·0	18·5
Enzyme solution + cathode liquid treated with 0·05 N-ammonia solution	12·6	19·5

* Cathode liquid evaporated to dryness, residue ignited, and ash dissolved in HCl, evaporated on water bath to dryness, then dissolved in water, neutralized and diluted to original volume.

Intestinal, faecal and liver phosphatase. Table 5 illustrates the effect of electrodialysis on these alkaline phosphatases. The enzyme activity/ml. remains almost constant for the first 20 hr.; then a sudden drop takes place. The cathode liquid showed a slight activating effect, but not as much as that noticed in the case of the kidney phosphatase. In some cases the cathode liquid was without any detectable activating effect, and the dialyzed enzyme lost its activity without any evidence of separation of a coferment, contrary to what was always found with the kidney phosphatase.

Table 5. *Effect of electrodialysis on intestinal, faecal and liver phosphatases, and the effect of the electrode chamber liquids on the enzyme activity*

	Activity (units/ml.)	
	No Mg	0·01 M-Mg
Intestinal phosphatase (+water, 1 : 1)	140	200
Electrodialysis against running distilled water, 1·5 increased to 10 ma.:		
After 2 hr.	140	200
„ 6 „	140	200
„ 10 „	130	200
„ 16 „	125	200
„ 20 „	115	180
„ 24 „	90	130
„ 28 „	40	55
„ 30 „	28	38
Enzyme solution + anode liquid (1 : 1)	18	27
Enzyme solution + cathode liquid (1 : 1)	36	45
Dialysis for further 4 hr.:		
Enzyme solution (+water 1 : 1)	0·4	0·4
„ (+anode 1 : 1)	0	0
„ (+cathode 1 : 1)	1·2	1·4
„ (+anode + cathode)	0	0
Faecal phosphatase solution (+water 1 : 1)	180	240
After 30 hr. electrodialysis against running distilled water, 1·5–10 ma.	65	95
Enzyme solution + anode liquid (1 : 1)	34	70
Enzyme solution + cathode liquid (1 : 1)	75	120
Liver phosphatase solution (+water 1 : 1)	96	120
After 20 hr. electrodialysis against running distilled water, 5 ma.	35	50
Enzyme solution + anode liquid (1 : 1)	15	22
Enzyme solution + cathode liquid (1 : 1)	45	65

On the other hand, the anode liquids showed a tendency to inhibit the intestinal, faecal and liver phosphatases. The pH of these liquids ranged from 2·3 to 4·7, and the inhibitory effect was more pronounced with the more acid solutions. The kidney enzyme was not sensitive to the same solution.

Effects of magnesium on phosphatases. Fig. 1 shows the effects of different magnesium concentrations on the activity of crude and purified intestinal and kidney phosphatase preparations. The enhancing

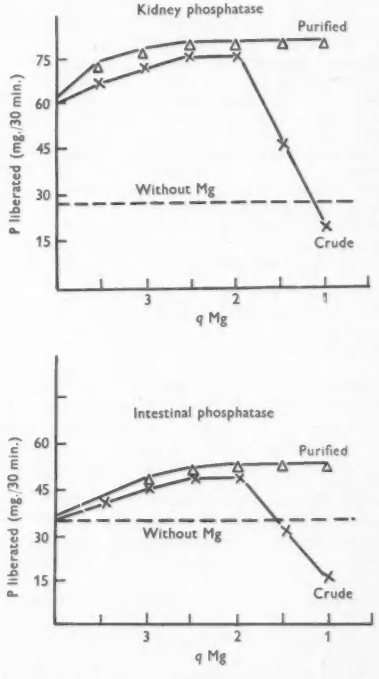

Fig. 1. Effect of Mg on crude and purified alkaline phosphatases. Hydrolysis of 0·005 M-sodium phenyl phosphate in carbonate-bicarbonate buffer, pH 10; $q\,\mathrm{Mg} = -\log[\mathrm{Mg}^{++}]$.

action of magnesium on kidney phosphatase is much more pronounced than that on the intestinal enzyme. With crude enzyme preparations there is an optimum concentration of magnesium beyond which the enzyme is inhibited. With purified enzyme

preparations, on the other hand, excess of magnesium has no such inhibitory effect.

Effect of amino-acids. Table 6 shows the effect of amino-acids on kidney and faecal enzymes. Both non-dialyzed kidney and faecal enzymes are activated by some amino-acids. The dialyzed kidney enzyme shows only feeble activation with cystine and cysteine, while glycine does not activate. The dialyzed faecal enzyme, on the other hand, shows a marked activation with amino-acids, that with histidine being especially noteworthy. This does not seem to be lessened with increasing concentrations of histidine up to 0·002 M. Cystine and cysteine, on the other hand, have no effect and tryptophan only a slight activating effect.

Table 6. *Effect of amino-acids on faecal and kidney alkaline phosphatase before and after dialysis*

(Ordinary dialysis in cellophan bags at room temperature against distilled water.)

	Activity (units/ml.)	
	No Mg	0·01 M-Mg
Non-dialyzed kidney phosphatase*	17·3	26·3
Glycine (0·002 M)	27·8	30·5
Cysteine (0·0005 M)	27·0	29·3
Histidine (0·001 M)	27·0	32·5
Cystine (0·001 M)	27·0	31·0
Dialyzed kidney phosphatase* (36 hr.)	9·0	10·5
Glycine (0·0002 M)	8·5	9·0
Cystine (0·005 M)	10·6	12·4
Cysteine (0·0005 M)	10·0	11·5
Non-dialyzed faecal phosphatase*	82·5	112·0
Glycine (0·0002 M)	96·0	125·0
Dialyzed faecal phosphatase* (36 hr.)	14·0	15·2
Glycine (0·0002 M)	35·7	46·5
Alanine (0·0005 M)	36·0	45·8
Cysteine (0·0005 M)	14·6	16·5
Cystine (0·0005 M)	14·6	16·5
Histidine (0·0005 M)	45·7	62·5
Histidine (0·001 M)	45·7	62·0
Histidine (0·002 M)	45·0	62·0
Tyrosine (0·001 M)	20·3	25·6
Tryptophan (0·001 M)	17·8	22·0

* Different preparations.

Effect of formaldehyde. Table 7 shows the effect of formaldehyde on the phosphatases obtained from different tissue autolysates. By inclusion of 0·1 ml. of 20 % neutral formaldehyde solution in the buffer-substrate-enzyme mixture (without previous incubation of the enzyme with the formaldehyde solution) a 95–100 % inhibition was attained with all tissue alkaline phosphatases, except those of intestinal mucosa and faeces, where the inhibition was 70 %.

Attempts were made to reactivate a kidney alkaline phosphatase that had lost most of its activity through formaldehyde treatment. Directly after adding the formaldehyde solution to the enzyme preparation a slight excess (over the theoretical to combine with the formaldehyde) of ammonia solution was added. The activity was then determined,

Table 7. *Effect of formaldehyde on the alkaline phosphatase of tissue extracts*

	Activity (units/100 ml.)		
	Without formaldehyde	Formaldehyde (0·5 %)	Inhibition (%)
Ox kidney	138·5	5	96
Human kidney	77·3	1·5	97
Adrenal glands	165	0	100
Dog bile diluted 1 : 200	28·5	0	100
Bone (young rabbit)	162	1·5	99
Dog faecal	75	24	68
Human intestinal	165	60	64
Liver	97	0	100
Pancreas	9	0	100
Spleen	120	0	100
Thyroid	47	0	100

and the effects of different amino-acids (glycine, alanine, histidine, arginine, tyrosine and tryptophan) as well as of the cathode liquid were tried. It was not possible to restore the activity by means of any of these substances.

DISCUSSION

These experiments support the hypothesis of Albers *et al.* (1938) and of Albers (1939) that phosphatase consists of an apo- and a co-enzyme. This is well illustrated with kidney phosphatase by a progressive loss of enzyme activity during electrodialysis, due to splitting off a factor (or factors) essential for enzyme activity which migrates towards the cathode. Thus the liquid in the cathode chamber shows strong reactivating properties when added to the holoferment which has been partially inactivated by electrodialysis. The liquid in the anode chamber, on the other hand, has no action on the enzyme activity. The anode and cathode liquids possess no enzymic activity either alone or combined.

The activating effect of the cathode liquid is mainly due to two factors: (A) magnesium (which is possibly replaceable by other metals), which can be easily split off and is responsible for the whole activating property of the cathode liquid during the early stages of electrodialysis, especially with weak currents; (B) an organic factor (possibly nitrogenous), which is not easily detachable from the apoenzyme, but splits off quickly with strong currents, or suddenly after prolonged ordinary dialysis. This factor withstands prolonged boiling, but is readily destroyed when treated with ammonia, excess alkali or acid. It is not identical with any of the amino-acids, since the latter do not exhibit a similar effect.

Magnesium seems to be essential for the activation of the holoferment. When the latter is carefully freed from magnesium and then added to the partially inactivated enzyme, it will produce no effect; but when magnesium is added as well the phosphomonoesterase activity of the mixture is much more than could be produced by adding magnesium alone. On the other hand, magnesium alone has no effect on the completely inactive enzyme, but in presence of the organic factors the activity is restored, although in no case to the initial level. Complete inactivation of the enzyme is probably accompanied by denaturation of the apoenzyme.

Under the conditions of these experiments this organic factor is essential for the reactivation of the dialyzed holoferment from ox kidney. It does not require any previous incubation, nor has it any inhibitory effect when present in excess. In contrast, it has no such action on the non-dialyzed kidney enzyme, nor on the electrodialyzed inactive enzyme prepared from liver or intestine. A possible explanation is that in the case of the liver and intestinal enzymes this factor is so strongly bound to the apoenzyme that the latter is inactivated by prolonged dialysis before any splitting of the coenzyme takes place.

These facts seem to indicate that the kidney phosphatase consists of three main components which are indispensable factors for the enzymic activity: a specific protein portion; a specific dialyzable organic group or groups; and magnesium. Magnesium produces its effect only in the presence of the dialyzable organic group or groups.

The two-metal theory involving zinc seems to be highly improbable since no evidence that zinc is indispensable has been obtained.

The behaviour of faecal, intestinal and liver phosphatases during dialysis has been found to differ from that of the kidney enzyme under the same experimental conditions.

The following are the results of a great number of experiments which have been carried out in this connexion:

(1) By ordinary dialysis in cellophan bags against distilled water, or feebly alkaline water at room temperature, it was observed that the kidney enzyme progressively decreased in activity. After 24 hr. dialysis the activity was greatly decreased, but it could be restored to the original level through the addition of optimum concentrations of magnesium. On further prolonged dialysis the activity still declined and after 48 hr. a marked loss of phosphatase activity took place. The enzyme was still activated by magnesium, but this could no longer restore the activity to its original value. The faecal, intestinal and liver enzymes, on the other hand, were markedly stable under the same conditions. Although a limited decrease in activity took place magnesium could still restore the original activity. Beyond this no further decline in activity took place, and the enzymes remained stable during 72 hr. dialysis in one experiment. With more prolonged dialysis activity was maintained for about 8–10 days at room temperature, following which it rapidly declined. This phenomenon can be explained on the basis of the previously mentioned postulate that the coenzyme, while very strongly bound to the apoenzyme in the case of faecal, intestinal and liver phosphatases, can be comparatively easily split in the case of the kidney enzyme.

(2) On electrodialysis the kidney enzyme was comparatively more stable than the other phosphatases. This could be partially accounted for, in many cases, by the marked sensitivity of these enzymes to anions. The anode liquid inhibited these enzymes, while it was quite harmless to the kidney enzyme. If the water circulating in the anode chamber was stopped during electrodialysis, the intestinal phosphatase was inhibited very quickly (even if dialysis was carried out at 0°), while the kidney enzyme, on the other hand, was not affected.

Although several factors have been known to influence the degree of activation of phosphatase by magnesium (e.g. concentration of magnesium, nature of substrate and its concentration, degree of enzyme purity, concentration and age, cf. Fischer & Greep, 1948), yet there have been strong indications that certain phosphatases are more activated by magnesium than others. Armstrong (1935) and Schmidt & Thannhauser (1943) described a 25% increase in the activity of faecal and intestinal phosphatases with optimum magnesium concentration. Drill, Annegers & Ivy (1944) also reported slight enhancement by magnesium of the elevated serum phosphatase during hepatic damage. Our findings concerning faecal, intestinal and liver phosphatases are in agreement with those of these authors. The kidney enzyme, on the other hand, has always been found to be much more activated by magnesium than either faecal, intestinal or liver phosphatases. This can again be explained on the basis of variation of affinity between the apoenzyme and its coenzyme and magnesium, in different phosphatases. When this affinity is very strong, the enzyme, as it naturally occurs, will be almost saturated with its requirements of magnesium, and hence the effect of any added amount will be only slight. On the other hand, when this affinity is weak, the enzyme will lose its magnesium readily during its extraction from the tissues and by mere ageing or by dilution, and thus the effect of added magnesium will be pronounced (cf. Fischer & Greep, 1948).

Amino-acids are shown here to activate both kidney and intestinal non-dialyzed enzymes. After prolonged dialysis of these enzymes, however, the effect of amino-acids on kidney enzyme was almost

negligible, while their strong activating influence was still persistent with the intestinal enzyme. This suggests that amino-acids exert their effect through certain groups in the coenzyme, which are easily split from the kidney enzyme while being relatively stable in the intestinal enzyme during dialysis.

The activation of kidney phosphatase by cysteine has been similarly reported by Williams & Watson (1940), who found that cysteine in low concentrations activates the alkaline phosphatase of bone, while with higher concentrations inhibition occurs.

On the other hand, Waldschmidt-Leitz, Schaffner & Bauer (1933), Bodansky (1936*a*, *b*) and Thannhauser, Reichel & Grattan (1937) reported that thiol compounds such as cysteine decrease the phosphatase activity.

With the dialyzed faecal phosphatase preparation in Table 6 both cysteine and cystine failed to reactivate the enzyme, while other amino-acids did so, especially histidine whose effect was marked up to a concentration of 0·002 M. Bodansky (1948) has recently reported the inhibitory effect of L-histidine on the activity of intestinal, bone and kidney phosphatases using higher concentrations (0·0064 M) than used here. He also pointed out that the basic amino-acids, L-histidine and L-lysine exert a greater inhibitory effect on rat-bone and kidney phosphatases than on intestinal phosphatase, whereas the reverse holds for the dicarboxylic L-glutamic acid.

The effect of formaldehyde on the different alkaline phosphatases provides a further distinction between kidney phosphatase and the intestinal and faecal enzymes. This phenomenon was previously noticed by Gould (1944). Bodansky (1937) also showed a difference between the intestinal phosphatase and other tissue phosphatases in connexion with their behaviour towards bile salts. The intestinal enzyme is less sensitive towards this inhibitor than the other phosphatases. It seems that the amino groups of the apoenzyme are essential for the phosphatase activity. Attempts to reactivate the formaldehyde-inhibited enzyme by addition of ammonium salts, amino-acids or cathode liquid were unsuccessful.

Owing to the fact that the kidney alkaline phosphatase has not yet been obtained in a satisfactory degree of purity, an investigation of the nature of the dialyzable coenzyme is not yet possible.

SUMMARY

1. The mechanism of enzyme inhibition during electrodialysis of faecal, intestinal, kidney and liver alkaline phosphatases has been studied.

2. The migration of a substance with strong reactivating properties towards the cathode during dialysis of the different phosphatases was most clearly demonstrable with the kidney enzyme. The properties of this substance have been investigated and compared with certain other substances.

3. No evidence in favour of the two-metal theory involving zinc could be obtained.

4. The presence of three main components seems to be indispensable for the normal alkaline phosphomonoesterase activity: a specific protein apoenzyme; an organic dialyzable coenzyme; and magnesium.

5. The activation of the enzyme by magnesium, and possibly certain other metals and amino-acids, takes place only in the presence of the dialyzable coenzyme.

6. The differences among the alkaline phosphatases of different origins are probably due to different affinities between the apoenzyme and its coenzyme, and possibly to slight variations in the nature of the apoenzyme in different phosphatases.

7. The apoenzyme appears to contain amino groups, which are essential for the phosphatase activity.

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The Action of Tyrosinase on Monophenols

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The many studies of tyrosinase have left unsolved the problem of the relationship between the so-called 'monophenolase' and 'catecholase' or 'polyphenolase' functions of this enzyme. It has seemed improbable that the same enzyme should effect two such dissimilar reactions as the introduction of a second hydroxyl group into the phenol ring and the dehydrogenation of an *o*-diphenol to give an *o*-quinone; yet the postulation of an independent specific monophenolase must remain under grave suspicion until fractions are obtained in which the relative monophenolase/catecholase activity is greater than in the crude mushroom juice. The alternative postulate, that the oxidation of monophenol is a secondary non-enzymic reaction, due to hydrogen peroxide or *o*-quinone formed from traces of *o*-diphenol which are presumed to be present, was first made by Onslow & Robinson (1928). It was criticized by Pugh (1929) and by Bordner & Nelson (1939), and a reconsideration of the evidence has led Nelson & Dawson (1944) to dismiss this possibility. The latter authors conclude that the monophenolase and catecholase functions are originally exercised by the same enzyme or enzyme complex, but that a centre in the enzyme which is essential for the monophenolase function, but not for the catecholase function, is (*a*) rather unstable towards the fractionation techniques which have been used, so that monophenolase activity is easily lost, and (*b*) inactive towards monophenol unless *o*-diphenol is being simultaneously oxidized by the enzyme, so that there is an induction period only relieved either by the slow build up in *o*-diphenol concentration initiated by spontaneous oxidation, or by the addition of *o*-diphenol to the system.

The first product of oxidation of monophenol is believed to be the corresponding *o*-diphenol, although the *o*-diphenol has only been isolated in the case of tyrosine → dihydroxyphenylalanine (Raper, 1926). The *o*-diphenol so formed is then also available as substrate to the enzyme, and may be oxidized to the quinone and beyond in the case of the primary substrates most studied (tyrosine, phenol, *p*-cresol) to a stage involving a further uptake of oxygen of 1 atom/mol. or more. The composition of the reaction mixture thus becomes quite complex with several different reactions proceeding simultaneously, but, in the studies of 'monophenolase' activity so far made, the monophenolase activity has been assessed in terms of the total oxygen uptake in this complex system. Keilin & Mann (1938) first reported the catalyzed oxidation of ascorbic acid in solutions containing tyrosinase and catechol, and Miller & Dawson (1941) have made use of this in their 'chronometric' method for measuring catecholase activity. The presence of ascorbic acid does not affect the catecholase activity of the enzyme, but *o*-quinone formed is then almost instantaneously reconverted to *o*-diphenol by the ascorbic acid, and is not detectable in the reaction mixture until all the ascorbic acid has been oxidized. This suggested that a study of the oxidation of monophenols by the enzyme in the presence of ascorbic acid might be of value. Under such conditions *o*-diphenol might be expected to accumulate, and the reaction mixture to remain uncomplicated by the accumulation of pigmented oxidation products of unknown constitution. The conditions would in fact be suitable for a study of the primary reaction, monophenol → *o*-diphenol. The present paper describes methods for the estimation of *o*-diphenol in reaction mixtures containing initially monophenol, tyrosinase and ascorbic acid; and results obtained with these methods in studies of the conversion of monophenol to *o*-diphenol in the cases of tyrosine, phenol, *p*-cresol and 4:5-dimethylphenol.* The experiments were carried out in the hope that the data obtained might throw more light on the vexed question of the mechanism of monophenol oxidation by tyrosinase.

METHODS

Enzyme preparation. The enzyme was prepared from the common cultivated mushroom, *Psalliota campestris*, by the method of Keilin & Mann (1938). The second fractional adsorption on $Ca_3(PO_4)_2$ in this procedure gave two fractions, the first of which, having Q_{O_2} (catechol)† and Q_{O_2} (*p*-cresol) values of *c.* 70,000 and 35,000, respectively, when freshly prepared, was used in all the experiments described in this paper. It was stored in the refrigerator under toluene, and had been so kept for 2 years when the work was begun. During this time its activity towards *p*-cresol had decreased by about one third, whilst the activity towards catechol appeared to have remained unchanged.

* This compound is strictly named '3:4-dimethylphenol'. The alternative name '4:5-dimethylphenol' is used throughout this paper to emphasize the relationship of the phenol to the *o*-diphenol, 4:5-dimethylcatechol, formed from it.

† All Q_{O_2} values are in μl. O_2/mg. dry wt./hr.

Conditions in tyrosinase experiments. The reaction mixtures in all cases contained 0·1 M-phosphate buffer (pH 7·0), and the temperature was 25°. The reactions were in general carried out in open vessels vigorously aerated by a stream of air for the provision of samples for *o*-diphenol and ascorbic acid estimation, and in the Warburg manometric apparatus with identical reaction mixtures, using a 2·00 ml. fluid phase and the standard technique, for the measurement of O_2 absorption. In the early experiments, with tyrosine as substrate, excellent agreement was obtained between the O_2 requirement calculated to correspond with the changes in ascorbic acid and *o*-diphenol concentration, and the O_2 uptake measured in parallel experiments. Later it was found in certain cases, viz. with phenol as substrate at the highest concentration used (0·1 M) and also with 4:5-dimethylphenol at quite low concentrations, that the oxidation of ascorbic acid and formation of *o*-diphenol was equivalent to a much smaller O_2 uptake than was observed in the parallel experiment. The difference could in neither case be attributed to a deficiency in aeration in the open vessel experiment, but, when this difference was observed, a rather persistent froth was always present above the surface of the reaction mixture in the open vessel, and the most likely explanation is that a major fraction of the enzyme was adsorbed in this froth and rendered ineffective by removal from the solution. To overcome this difficulty when it arose, manometric experiments only were carried out, in multiplicate. Individual manometers were removed from the bath at different times after the beginning of the reaction, and their reaction mixtures immediately analyzed for ascorbic acid and *o*-diphenol. Composite curves were constructed from the data to show the changes in the reaction mixture with time.

Estimation of o-diphenols

It was desired to estimate ascorbic acid as well as *o*-diphenol in colourless reaction mixtures containing the corresponding monophenol, tyrosinase, ascorbic acid and 0·1 M-phosphate buffer. The reaction was always stopped at the appointed time by pipetting a measured sample into an equal volume of 4% metaphosphoric acid and the protein-free filtrate was used for both *o*-diphenol and ascorbic acid estimations. The following account of methods of *o*-diphenol estimation refers, therefore, only to estimations in this particular type of filtrate. The standard solutions of *o*-diphenols, used for the preparation of calibration curves, contained the same amounts of phosphate buffer and metaphosphoric acid as are present in such a filtrate. In no case was the presence of monophenol found to have any affect on the diphenol estimation. Ascorbic acid was only added to the standard solutions when it was found materially to affect the rate of development of the colour which was the basis of the method of estimation.

A. 3:4-*Dihydroxyphenylalanine* (DOPA). Schild (1933) and von Euler (1933) used the red colour developed on oxidation with I_2 for the colorimetric estimation of adrenaline. Evans & Raper (1937*a*) found that a similar method could be used for the estimation of DOPA. The method adopted in the present work was essentially that of Evans & Raper, improved by the use of the Spekker photoelectric absorptiometer instead of visual colour matching. A calibration curve was first prepared. The solution required for establishing a point on the curve was made by mixing in the order given: (*a*) 8·0 ml. of standard DOPA solution, (*b*) 14·0 ml. of 0·5 M-phosphate buffer (pH 6·0), (*c*) 10·0 ml. of 0·192 N-NaOH and (*d*) 3·8 ml. of 0·1 N-I_2. This mixture gave a final pH of 6·0. Ninety seconds after the I_2 addition (*e*) 4·2 ml. of 0·1 N-$Na_2S_2O_3$ were added, and the mixture immediately transferred to the 4 cm. absorptiometer cell. Absorptiometer readings were taken, with a blue filter (no. 6 of Hilger set H455), at timed intervals for a period of *c.* 5 min. The red colour remaining in the solution after the $Na_2S_2O_3$ addition, due to the presence of DOPA, was found to diminish appreciably in intensity even within this time. The logarithms of the absorptiometer readings were plotted against time after $Na_2S_2O_3$ addition, and the linearity of the plot permitted extrapolation back to zero time. By following the same procedure with the enzyme reaction filtrate, using the latter instead of (*a*) above, a zero-time absorptiometer reading was obtained and the corresponding DOPA concentration read on the calibration curve. The latter covered DOPA concentrations up to 0·20 mg./ml.

When this method of estimation was applied to samples taken at intervals from an aerated reaction mixture containing initially tyrosinase, ascorbic acid and DOPA (0·2 mg./ml.) but no tyrosine, the DOPA concentrations found 0·5, 5, 10, 15, 20, 30, 40, 50, 60 and 70 min. after the beginning of the enzyme reaction were respectively 0·207, 0·207, 0·206, 0·208, 0·206, 0·207, 0·206, 0·207, 0·204 and 0·208 mg./ml. These figures vouch for the reliability of the method, and for the absence of interference by ascorbic acid, the concentration of which fell from 4·0 to 0·2 mg./ml. during the experiment. They also illustrate the efficiency with which ascorbic acid reconverts DOPA quinone into DOPA.

B. *Catechol.* The molybdate method, used by Rae (1930) for adrenaline estimation and said to be applicable to all catechol derivatives, was considered. The statement of Evans & Raper (1937*a*) that ascorbic acid gives a green-blue coloration with the molybdate reagent was discouraging, but trial did not reveal any trace of such coloration, and the following procedure was found to be applicable even in the presence of ascorbic acid. For the determination of a point on a calibration curve a solution was made up by mixing (*a*) 4·0 ml. of catechol standard solution, (*b*) 7·0 ml. of 0·5 M-phosphate buffer (pH 6·0), (*c*) 5·0 ml. of 0·192 N-NaOH and (*d*) 2·0 ml. of 20% ammonium molybdate. An absorptiometer reading was taken 20 min. after the molybdate addition, using the 1 cm. cell and filter no. 7 of Hilger set H 455. The colour intensity continues to increase after its first almost instantaneous development, but not so rapidly (e.g. absorptiometer reading increasing from 0·246 at 20 min. to 0·261 at 150 min.) as to lead to error, provided that the interval between molybdate addition and absorptiometer reading is kept within the limits of 20–30 min. A calibration curve was constructed covering catechol concentrations up to 0·8 mg./ml. To increase the accuracy of the method at the lower concentrations a second curve was prepared using the 4 cm. absorptiometer cells with doubled volumes of solutions (*a*) to (*d*) above. This covered a range up to 0·3 mg./ml. For the estimation of catechol formed from phenol in enzymic reaction mixtures, the metaphosphoric acid filtrates of the latter were used in place of solution (*a*) in the procedure which has been described.

C. *Homocatechol* (4-*methylcatechol*). When a beginning was made with the preparation of a calibration curve for this estimation by the procedure used for catechol, it was noticed that the solutions prepared for absorptiometer examination,

left standing at room temperature for a day or two, increased in colour intensity by a factor of about 3. The possibility of increasing the sensitivity of the estimation by delaying the colour measurement until this increase had reached its maximum was obvious. Ascorbic acid was found not to affect significantly the colour intensity developing within 20 min. of the addition of molybdate, but it markedly delayed the subsequent increase in colour in such a way as to suggest that the onset of a phase of relatively rapid increase waited upon the exhaustion of the ascorbic acid by spontaneous oxidation. Independently of the amount of ascorbic acid present, the maximum colour finally reached was constant for a given homocatechol concentration and was stable for 2–3 days.

For the preparation of the calibration curves finally used, ascorbic acid was added to the standard homocatechol solutions in an amount equal to the maximum to be expected in the enzyme reaction mixtures (4 mg./ml.). The procedure was the same as in the estimation of catechol, but absorptiometer readings were taken 20 min. and 3 hr. after molybdate addition and each day until the maximum colour was attained (not more than 5 days). The filter used was Ilford spectrum violet no. 601 which was found to give readings *c.* 50% greater than the filter previously used. Two calibration curves were drawn, one from the 20 min. readings covering concentrations up to 1·5 mg./ml., the other from the maximum readings for concentrations up to 0·3 mg./ml. only. The latter curve was used unless homocatechol concentrations outside its range, giving maximum colours too intense for satisfactory measurement in the absorptiometer, were encountered. In the concentration range 0·2–0·3 mg./ml., in which a reliable comparison could be made between assessments based on the 20 min. and on the maximal colour readings, good agreement was found.

D. 4:5-*Dimethylcatechol.* This diphenol behaved very like catechol in the molybdate reaction under the conditions which have been described. For the estimation of 4:5-dimethylcatechol formed enzymically from 4:5-dimethylphenol the procedure was the same as for the homocatechol estimations.

Estimation of ascorbic acid

Samples of the metaphosphoric acid filtrates of the reaction mixtures, which had been prepared as already described for *o*-diphenol estimation, were titrated with a standardized solution of 2:6-dichlorophenolindophenol from a microburette.

RESULTS

Oxidation of tyrosine

Course of oxidation in presence of ascorbic acid. The results were of the type illustrated in Fig. 1. It will be seen that the observed oxygen uptake was in good agreement with that calculated for the oxidation of ascorbic acid and the formation of DOPA. The lag phase at the beginning of the oxygen-uptake curve corresponds with the lag in the initiation of ascorbic acid oxidation. Since the latter is a secondary oxidation effected by DOPA quinone, as the concentration of DOPA and, therefore, the rate of DOPA-quinone formation increases from zero, the rate of ascorbic acid oxidation also increases. In this experiment it reaches a maximal value when the DOPA concentration has reached 0·0005 M, and remains almost linear until all the ascorbic acid has been oxidized. The curve in Fig. 1, showing the changing concentration of DOPA during the experiment, provides no evidence of the occurrence of a lag phase in the oxidation of tyrosine to DOPA.

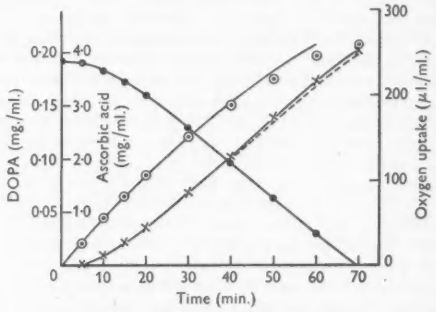

Fig. 1. Oxidation of tyrosine by tyrosinase in the presence of ascorbic acid. Solution saturated with tyrosine, excess solid present. Enzyme concentration, 0·07 mg. (dry wt.)/ml. ⊙—⊙, dihydroxyphenylalanine concentration, the curve representing the expression $Q = 0{\cdot}77t/(160+t)$, where Q = concentration and t = time; ●—●, ascorbic acid concentration; ×—×, oxygen uptake, the broken line showing the calculated oxygen requirement for ascorbic acid oxidation and DOPA formation.

Decline in rate of formation of DOPA. The rate of DOPA accumulation appears to be maximal at the start, and then progressively declines, although the concentration of DOPA continues to increase so long as ascorbic acid is present to prevent its oxidation beyond the reversible DOPA-quinone stage. Since the concentration of the substrate of the primary oxidation (tyrosine: saturated solution with solid phase present) remains constant throughout the experiment, the decline in rate of DOPA formation must be due either to inactivation of enzyme or to inhibition by reaction products, i.e. DOPA or dehydroascorbic acid. In the absence of any other indication of the second of these alternatives, the first seems the more probable, the susceptibility of tyrosinase to inactivation during its reactions being widely recognized.

Calculation of Q_{O_2} *for oxidation of* DOPA. On the assumption that the decrease in rate of DOPA formation is due solely to enzyme inactivation this rate at any given time, i.e. the slope of the tangent to the DOPA concentration curve at that time, may be taken as a measure of the amount of active enzyme still present. By drawing tangents to the ascorbic acid concentration curve the rate of oxidation of ascorbic acid at any moment may be determined, and this is equal to the rate of oxidation of DOPA to

DOPA quinone. From this, by the use of the figure for the amount of residual active enzyme at the same moment obtained in the manner indicated above, one can calculate a Q_{O_2} value for the oxidation of DOPA by the enzyme preparation used in the experiment. The Q_{O_2} values so obtained for different times after the beginning of the reaction each relate to a different substrate (DOPA) concentration, the magnitude of which may be found from the DOPA-concentration curve.

Thus the data required for the construction of an activity-substrate concentration curve can be abstracted, the activity referred to being the catecholase (as distinct from the monophenolase) activity of the enzyme, with DOPA as substrate. This curve may then be compared with the corresponding curve determined in direct experiments with the enzyme and DOPA; if the two curves coincide, the above interpretation of events in the reacting system would be strongly supported.

Rate of DOPA *formation at zero time.* In attempting to treat the experimental data in the above way, a primary difficulty is in the satisfactory assessment of the rate of DOPA formation at zero time, before any enzyme inactivation has taken place. For the oxidation of catechol by tyrosinase in the presence of ascorbic acid Miller & Dawson (1941) have shown that the experimental data fit the relationship $Q = at/(b+t)$, where Q is the total substrate which has been oxidized in time t, and a and b are constants, the magnitude of b being determined by the rate of inactivation of the enzyme. The reciprocal of Q plotted against the reciprocal of t gives a straight line, and as the slope of this line is the reciprocal of the initial reaction velocity (dQ/dt for $t=0$) the latter may readily be determined with considerable accuracy.

Applying this device in the instance under discussion, it is found that the DOPA-concentration curve during the greater part of the experiment may be represented reasonably well by the expression $Q = 0{\cdot}77t/(160+t)$, in which Q is the DOPA concentration in mg./ml. and t is the time in minutes after the start of oxidation. The values of Q calculated from this expression are compared with the observed values in columns 2 and 3 of Table 1. The fourth column of Table 1 gives the rates of DOPA formation at different times, i.e. the calculated values of $dQ/dt = 123/(160+t)^2$, these being taken as a measure of the amount of enzyme still in the active condition. The fifth column shows this amount as a percentage of the amount initially present. The Q_{O_2} values for ascorbic acid oxidation, representing DOPA oxidation by the residual active enzyme, are in column 6, and the corresponding DOPA concentrations in molar terms in the last column of the table.

Table 1. *Oxidation of tyrosine + ascorbic acid by tyrosinase*

Time (min.)	DOPA concentration (mg./ml.) Found	Calc.*	Rate of DOPA formation (mg./ml./min.)†	Enzyme still active (%)	Q_{O_2} of ascorbic acid oxidation	DOPA concentration (10^{-4} M)
0	0·00	0·00	0·0048	100	—	—
5	0·021	0·023	0·0045	94	—	1·2
10	0·045	0·045	0·0043	90	2400	2·3
15	0·065	0·066	0·0040	83	3000	3·3
20	0·085	0·086	0·0038	79	3700	4·3
25	—	0·104	0·0036	75	4500	5·2
30	0·120	0·122	0·0034	71	5000	6·2
40	0·150	0·154	0·0031	66	5600	7·8
50	0·175	0·183	0·0028	58	6150	9·3
60	0·197	0·210	0·0025	52	6800	10·7

* Calculated from $Q = 0{\cdot}77t/(160+t)$.
† Calculated from $dQ/dt = 123/(160+t)^2$.

Reaction velocity and DOPA *concentration.* In a separate series of manometric experiments the relationship between reaction velocity and substrate (DOPA) concentration was studied directly. DOPA itself in known concentration without tyrosine was used as substrate, and ascorbic acid and buffer concentrations were the same as in the experiments

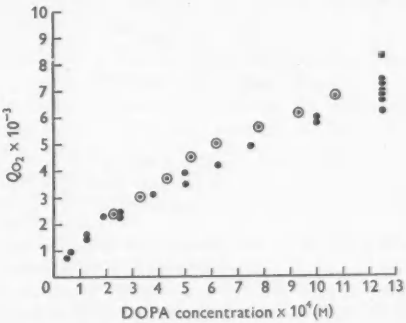

Fig. 2. Oxidation of DOPA by tyrosinase. Enzyme activity-substrate concentration curve: ●, direct determinations; ⊙, calculated from data of tyrosine oxidation experiment.

already described. The oxygen uptake during the first 5 min. after the addition of the enzyme to the DOPA-ascorbic-buffer mixture was used for the purpose of calculating the Q_{O_2} value. The Q_{O_2} values obtained by this direct method are plotted against substrate concentration in Fig. 2, together with the values of Table 1 calculated from the data of the tyrosine oxidation experiment. The two sets of values are seen to be consistent with each other, and this lends support to the interpretation of the tyrosine oxidation experiment which has been put forward.

rate continued to increase until in saturated (*c.* 0·025 M) DOPA solution Q_{O_2} values of about 25,000 were recorded.

Q_{O_2} of true monophenolase and of the overall reaction. The initial rate of DOPA formation in the experiment represented in Fig. 1, calculated from the reciprocal plot of the DOPA-accumulation curve, was 0·0048 mg./ml./min., which corresponds to a Q_{O_2} (μl. O_2/mg. dry wt./hr.) of 230 for the enzyme preparation used. When tyrosine in saturated solution in 0·1 M-phosphate buffer (pH 7·0) was oxidized in the absence of ascorbic acid, with the same enzyme

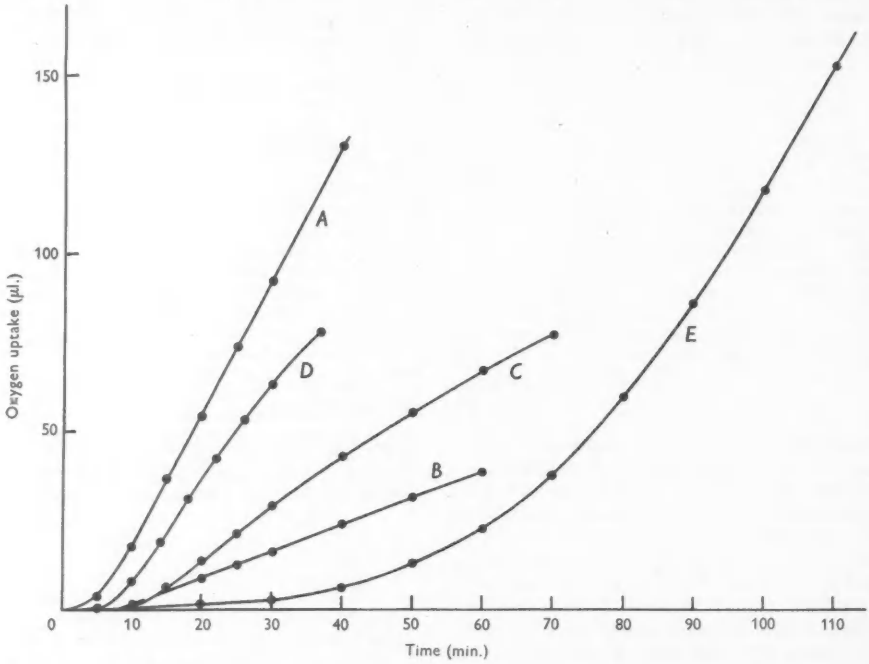

Fig. 3. Oxidation of monophenols with tyrosinase, in the absence of ascorbic acid. Curves *A* and *B*, tyrosine, saturated solution (*c.* 0·0025 M); *A*, 0·35 mg. enzyme; *B*, 0·07 mg. enzyme. Curve *C*, 0·005 M-phenol, 0·02 mg. enzyme. Curve *D*, 0·005 M-*p*-cresol, 0·007 mg. enzyme. Curve *E*, 0·017 M-4:5-dimethylphenol, 0·35 mg. enzyme.

It follows from this result that the presence of tyrosine in saturated solution (*c.* 0·0025 M) does not significantly inhibit the oxidation of DOPA by the enzyme. The apparently linear course of the oxidation of ascorbic acid seen in Fig. 1 after the maximal rate is reached must be ascribed to a fortuitous balance of two opposing factors, viz. the progressive inactivation of the enzyme which would of itself diminish, and the progressive increase in DOPA concentration which would of itself increase, the reaction rate. With continued increase in DOPA concentration above the range of Fig. 2 the reaction

preparation, the oxygen uptake followed the curves shown in Fig. 3. After the induction period the rate of uptake became nearly linear at values of 3·82 and 0·75 μl./min. at the two enzyme concentrations employed, one of which was five times the other. The corresponding Q_{O_2} values are 650 and 640, and two other similar determinations gave values of 650.

These Q_{O_2} values of 230 for the true monophenolase activity and 650 for the overall reaction are in accordance with the relative speeds of the component steps in the oxidation of tyrosine suggested by Evans & Raper (1937*b*). According to the scheme put

forward by these authors the primary slow oxidation to DOPA, requiring one atom of oxygen/mol. of tyrosine, is followed by three successive relatively fast reactions, two of which, the conversion of DOPA to DOPA quinone, and, after ring closure, the oxidation of 5:6-dihydroxydihydroindole-1-carboxylic acid to its quinone, each require one atom of oxygen. If the secondary reactions are markedly faster than the first reaction, one would then expect the Q_{O_2} for the whole oxidation to be approximately three times the Q_{O_2} of the isolated primary reaction. The activity-substrate concentration curve of Fig. 2 shows that the Q_{O_2} for DOPA oxidation reaches a value of 230 at a DOPA concentration of *c.* 0·0002 M. During the oxidation of tyrosine by tyrosinase in the absence of ascorbic acid one would, therefore, not expect DOPA to accumulate beyond this concentration, at which rate of removal would balance rate of formation; and the difficulty experienced by earlier workers (Raper, 1926) in attempting to demonstrate DOPA formation by isolation is consequently not surprising.

Oxidation of phenol

Rate of formation of catechol in presence of ascorbic acid. The results obtained with phenol were, except in one respect, qualitatively similar to the results of the tyrosine experiments. The course of catechol accumulation at the three phenol concentrations studied is shown in Fig. 4. The curves provide no evidence of an induction period in the primary oxidation of phenol to catechol. The catechol concentrations which had been built up when the experiments were terminated by the exhaustion of the ascorbic acid were 0·0053 M from 0·1 M-phenol, 0·0021 M from 0·01 M-phenol and 0·00095 M from 0·001 M-phenol, representing a conversion of 5, 21 and 95 % respectively of the substrate initially present. Only in the case of the lowest phenol concentration is the fall in primary substrate concentration during the course of the experiment likely to have been a significant factor in bringing about the slowing down of catechol formation, and the form of the catechol accumulation curves at the higher phenol concentrations is attributed to the result of enzyme inactivation. These curves can be represented reasonably well by the equations $Q = 2{\cdot}0t/(122+t)$ and $Q = 0{\cdot}53t/(38+t)$; the lines drawn in Fig. 4 are the plots of these equations and show the closeness of fit.

The equations give initial Q_{O_2} values for the isolated primary oxidation of 2900 with 0·1 M- and 2400 with 0·01 M-phenol and in other similar experiments figures of 3100 and 2700, respectively, were obtained. In the experiment with 0·001 M-phenol the initial Q_{O_2} relating to this concentration cannot be judged with confidence because of the rapid fall in substrate concentration. The use of the first two points only of the catechol-accumulation curve by the reciprocal plot method gives a Q_{O_2} of 1900. When phenol was presented as substrate to the same enzyme preparation in the absence of ascorbic acid, the initial lag in oxygen absorption was much more pronounced and the uptake followed a course such as that shown in Fig. 3. The maximal rate of oxygen usage, reached after 20 min., corresponds to a Q_{O_2} of 4700, which is of the order of magnitude to be expected if the rate of the primary reaction determines the overall oxygen uptake rate.

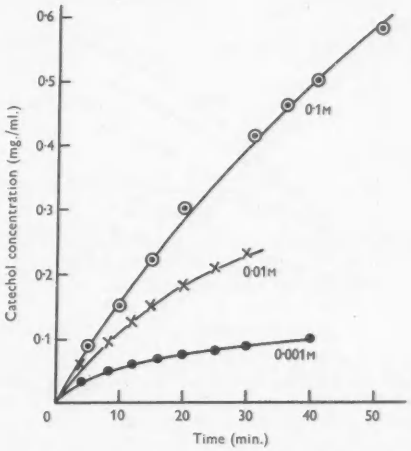

Fig. 4. Accumulation of catechol during the oxidation of phenol by tyrosinase in the presence of ascorbic acid. The initial phenol concentration is indicated alongside each curve. Enzyme concentration, 0·035 mg./ml. The curve drawn for 0·1 M-phenol represents the expression $Q = 2{\cdot}0t/(122+t)$, and that for 0·01 M-phenol the expression $Q = 0{\cdot}53t/(38+t)$, Q being catechol concentration in mg./ml.

Inhibition of catechol oxidation by phenol. The results obtained in the experiments with phenol differed qualitatively from those given by tyrosine in one respect. Analysis of the results with tyrosine produced no evidence that tyrosine had any inhibitory effect on the oxidation of DOPA by the enzyme. The results of a similar analysis of the data of the phenol experiments, however, could only be satisfactorily interpreted if an inhibitory effect on catechol oxidation were attributed to phenol. It is not proposed to give this analysis in detail, since the same effect is seen in a more intense form during the oxidation of 4:5-dimethylphenol, to be described later. The inhibitory effect of phenol on catechol oxidation was demonstrated in direct experiments such as that illustrated in Fig. 5. The inhibition was competitive in type. When catechol and phenol were present in equimolar proportions the phenol was

without significant effect. But when the relative catechol concentration was reduced to 1/5 there was marked inhibition, and at 1/20 strong inhibition, of the initial oxygen-uptake rate. It must be borne in mind that in the phenol-containing reaction mixtures the concentration of catechol increases from the moment at which the enzyme is added, owing to the conversion of phenol to catechol by the enzyme. As a result of the increase in catechol concentration and the decrease in phenol concentration, the inhibition might be expected to pass off with time, and that it does so is evident in the curves.

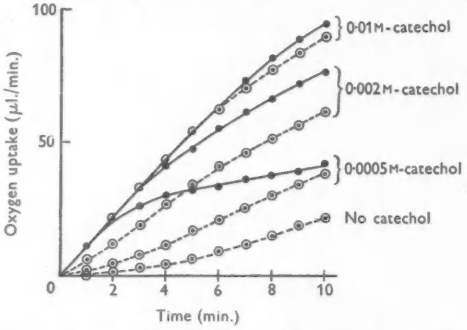

Fig. 5. Effect of phenol on oxidation in the ascorbic acid + catechol + tyrosinase system. ●—●—●, without phenol; ⊙—⊙—⊙, with 0·01M-phenol.

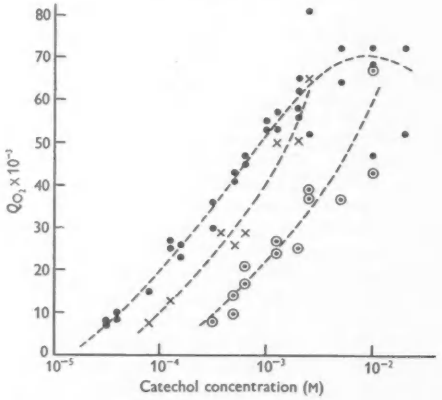

Fig. 6. Oxidation of catechol by tyrosinase. Effect of phenol on the enzyme activity-substrate concentration curve. Dots, no phenol present; crosses, with 0·001M-phenol; circles, with 0·01M-phenol.

Q_{O_2} of catechol oxidation. The Q_{O_2} values obtained in a series of determinations similar to those shown in Fig. 5 and using reaction mixtures with various catechol concentrations and (*a*) no phenol, (*b*) 0·001M-phenol and (*c*) 0·01M-phenol are plotted against catechol concentration in Fig. 6. The Q_{O_2} values are based on the oxygen uptake in the first 2 min. of the reaction and are admittedly subject to a rather large possibility of experimental error, and this is evident in the scatter of points in Fig. 6. But the nature of the relationship between enzyme activity and catechol concentration, and the competitive inhibitory effect of phenol, are clearly discernible. One half the maximal activity is reached at *c.* 0·0003M-catechol in the absence of phenol and at *c.* 0·0007 and 0·002M-catechol in the presence of 0·001 and 0·01M-phenol respectively.

Oxidation of p-*cresol*

Course of homocatechol formation in presence of ascorbic acid. The course of homocatechol accumulation in a series of experiments at different *p*-cresol

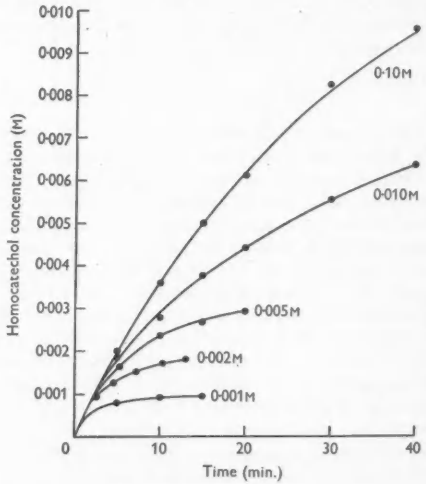

Fig. 7. Accumulation of homocatechol during oxidation of *p*-cresol with tyrosinase in the presence of ascorbic acid; enzyme concentration, 0·035 mg. (dry wt.)/ml. The initial *p*-cresol concentrations in the various experiments are recorded at the ends of the curves. The uppermost curve represents the expression $Q = 2{\cdot}5t/(45{\cdot}6 + t)$.

concentrations is shown in Fig. 7. The enzyme concentration was the same as in the experiments with phenol, but the formation of *o*-diphenol was much faster. With the smaller *p*-cresol concentrations, the asymptotic approach to complete conversion to homocatechol is apparent and even with 0·01M-*p*-cresol 64% conversion had taken place in 40 min. Only at the highest *p*-cresol concentration (0·1M) is the resultant decrease in *p*-cresol sufficiently small relative to the initial concentration to make it

unlikely that the rate of the primary oxidation is affected during the course of the experiment by the fall in substrate concentration.

The uppermost curve in Fig. 7 is the curve of the expression $Q = 2{\cdot}5t/(45{\cdot}6+t)$, where Q is the homocatechol concentration in mg./ml. This expression fits the points for the experiment with 0·10 M-*p*-cresol very well. The initial reaction velocity ($t=0$) is 0·55 mg./ml./min. corresponding to a Q_{O_2} of 8500. Using the homocatechol formation in the first 5 min. for the calculation of Q_{O_2} values to represent the monophenolase activity of the enzyme in each case, the results shown in Table 2 were obtained. The *p*-cresol concentrations are the means of the values at the beginning and at the end of the 5 min. period. The figures suggest that half the maximal reaction velocity is reached at a substrate concentration of about 0·001 M.

Table 2. *Oxidation of* p-*cresol+ascorbic acid by tyrosinase: monophenolase activity at various substrate concentrations*

p-Cresol concentration (M)	Monophenolase Q_{O_2}
0·098	7880
0·0096	7200
0·0046	6200
0·0013	5180
0·0006	3100

Q_{O_2} of overall oxidation. When *p*-cresol in the absence of ascorbic acid is oxidized by excess of tyrosinase, the oxygen uptake approaches three atoms/molecule of *p*-cresol. If the overall rate of the reaction is determined by the rate of the primary oxidation of *p*-cresol to homocatechol, one would expect it, in terms of oxygen-uptake rate, to be about three times that of the primary oxidation. The course of oxygen uptake when the enzyme preparation used in this series of experiments oxidized *p*-cresol in the absence of ascorbic acid is shown in Fig. 3. The maximal rate, reached after 10 min., corresponds to a Q_{O_2} of 25,000, which is of the order of magnitude expected in relation to the figure of about 8500 found for the isolated primary reaction.

Q_{O_2} of homocatechol oxidation. Inhibition by monophenol. Using the rate of ascorbic acid oxidation in these experiments as an index of the rate of homocatechol oxidation, Q_{O_2} values were calculated in the manner already given in the description of the tyrosine experiments. They were found to be lower than the values obtained in direct determinations with homocatechol as substrate at equivalent concentrations. As in the case of phenol-catechol, this suggested the operation of an inhibitory effect of the monophenol on the oxidation of the corresponding *o*-diphenol, which was verified by direct experiment (Fig. 8).

Oxidation of 4:5-*dimethylphenol+ascorbic acid*

Rate of accumulation of 4:5-*dimethylcatechol.* This was found by the reciprocal plot method to conform well with the relationships $Q = 0{\cdot}76t/(49+t)$ and $Q = 1{\cdot}0t/(115+t)$ for initial monol concentrations of

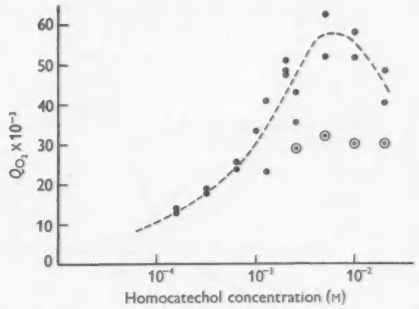

Fig. 8. Oxidation of homocatechol by tyrosinase. Enzyme activity-substrate concentration curve. ⊙ ⊙, Q_{O_2} values determined in the presence of 0·01 M-*p*-cresol.

0·02 and 0·005 M respectively, Q being the 4:5-dimethylcatechol concentration in mg./ml./min. after time t. The initial rates of diol formation in the two cases (dQ/dt, $t=0$) correspond to Q_{O_2} values of 330 and 180. No attempt was made to treat the data obtained at lower initial monol concentrations in this way; the method of estimation of the diol is not sufficiently reliable at lower concentrations, and there is the additional complication of a relatively rapidly changing monol concentration. At 0·001 M, the latter had been reduced to half in about 20 min.

Q_{O_2} for diphenol oxidation in presence and absence of the monophenol. In the experiments at the lower 4:5-dimethylphenol concentrations, the lag in the initiation of ascorbic acid oxidation and oxygen absorption was much more prolonged, and the subsequent acceleration much more dramatic, than in any of the experiments with the other substrates. The general nature of the difference may be appreciated if Fig. 9, showing the results of an experiment with 0·001 M-4:5-dimethylphenol, is compared with Fig. 1. The results of an analysis of the data of Fig. 9 are recorded in Table 3. The rates of ascorbic acid oxidation given in the table were obtained from the slopes of tangents drawn to the ascorbic acid concentration curve at the appropriate points. They are taken to be equivalent to the rates of oxidation of 4:5-dimethylcatechol and the Q_{O_2} (catecholase) values given in the third column of the table are based on them. The dimethylcatechol concentrations in column 4 are taken from the accumulative curve of Fig. 9.

The rate of oxidation of ascorbic acid during the first 20 min. of this experiment was so small as to be within the range of spontaneous oxidation under these conditions in controls without enzyme, so that even the very small Q_{O_2} values recorded in this period must have been seriously overestimated. The later Q_{O_2} values, on the other hand, would be subject to a progressively greater magnification if it were possible to relate them to the amount of residual

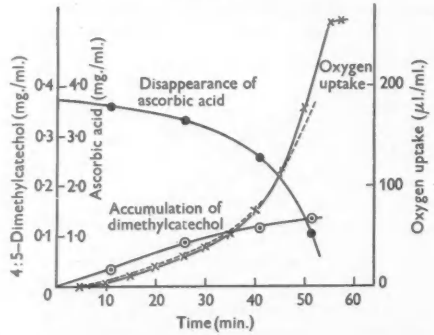

Fig. 9. Oxidation of 0·001 M-4:5-dimethylphenol by tyrosinase in the presence of ascorbic acid. Enzyme concentration, 0·115 mg. (dry wt.)/ml. The broken line shows the calculated oxygen requirement for ascorbic acid oxidation and dimethylcatechol formation.

active enzyme instead of to the amount of enzyme initially present. These considerations together indicate that the real increase in Q_{O_2} (catecholase) must have had a more sudden onset than the figures in Table 3 suggest. Since, in the absence of 4:5-dimethylphenol, Q_{O_2} values for the oxidation of 4:5-dimethylcatechol were found to be of the order of 40,000 at a dimethylcatechol concentration such as had been reached within less than 10 min. of the beginning of the experiment under discussion (Fig. 10), the delay in the attainment of a high Q_{O_2} must be due to the inhibitory effect of the dimethylphenol present.

The acceleration of ascorbic acid (=dimethylcatechol) oxidation is attributed to (*a*) the accumulation of the substrate 4:5-dimethylcatechol and (*b*) the removal of the inhibitory effect of the monol on the oxidation of the diol, as the relative concentration of these changes. It is most marked when the conversion of monol to diol is approaching completion and the monol/diol ratio changing rapidly to

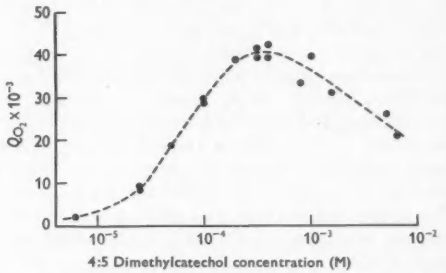

Fig. 10. Oxidation of 4:5-dimethylcatechol by tyrosinase; enzyme activity-substrate concentration curve.

very small values. The change in this ratio is shown in the last column of table 3. At higher initial dimethylphenol concentrations, e.g. 0·02 M, the molar ratio monol/diol remained greater than unity and the inhibitory effect of monol on the diol oxidation was maintained throughout the experiment.

The course of oxygen uptake in manometric experiments in which smaller concentrations of 4:5-dimethylphenol were used is seen in Fig. 11. In those curves which relate to primary substrate concentrations of less than 0·002 M the very slow initial uptake and subsequent rapid acceleration are strikingly evident. The initial rate is scarcely measurable, and of an order of magnitude accountable in terms of the monophenolase activity of the enzyme and the autoxidation of ascorbic acid. For a given enzyme concentration, the lower the concentration of dimethylphenol the earlier and the more sudden is the onset of the rapid phase of oxygen absorption; maximal rates with 0·0004, 0·0001 and 0·000025 M-dimethylphenol are reached respectively in 80, 22 and 8 min. (Fig. 11*b*). On the other hand, at a given substrate concentration the rapid phase appears earlier, the greater the enzyme concentration; with 0·0004 M-dimethylphenol, at 10–15 min.

Table 3. *Oxidation of 4:5-dimethylphenol + ascorbic acid by tyrosinase*

(Dimethylphenol concentration initially 0·001 M.)

Time (min.)	Rate of ascorbic acid oxidation (μl. O_2/ml./min.)	Q_{O_2}* catecholase	Dimethylcatechol concentration (10^{-3} M)	Dimethylphenol concentration (10^{-3} M)	Molar ratio: monol/diol
10	0·8	610	0·26	0·74	2·9
20	1·3	670	0·49	0·51	1·0
30	2·3	1200	0·69	0·31	0·45
40	5·1	2600	0·84	0·16	0·19
50	12·4	6400	0·94	0·06	0·06

* Not corrected for inactivation.

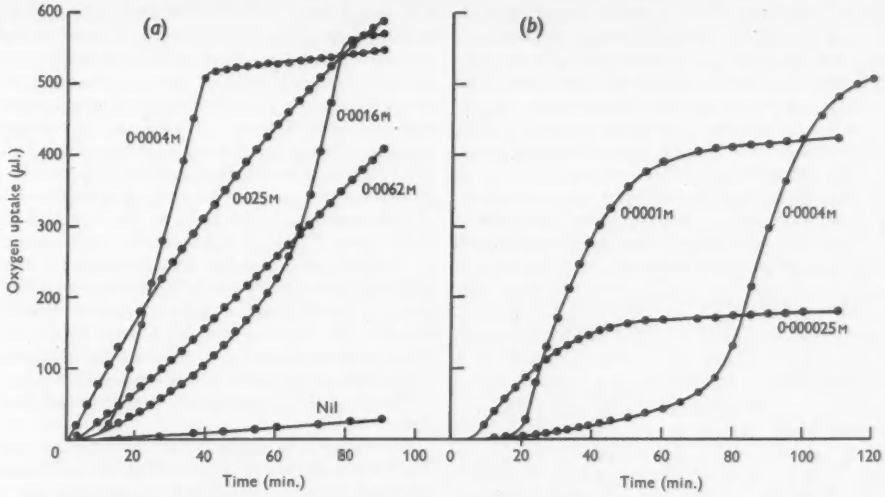

Fig. 11. Oxidation of 4:5-dimethylphenol + ascorbic acid by tyrosinase. Reaction mixture vol. 2·0 ml., containing 8 mg. ascorbic acid (≡508 μl. O_2). Initial dimethylphenol concentrations as stated against the curves; (*a*) 0·23 mg., (*b*) 0·058 mg., tyrosinase (dry wt.).

with 0·232 mg. enzyme (Fig. 11*a*), but at 70–80 min. with 0·058 mg. enzyme (Fig. 11*b*). This is in agreement with the interpretation already put forward, that the catecholase function of the enzyme is strongly inhibited by the monophenol, the inhibition passing off swiftly when the ratio monophenol/diphenol falls rapidly as the conversion of the one to the other approaches completion. At the higher dimethylphenol concentrations, 0·025 and 0·0062M (Fig. 11*a*), the rapid phase of oxygen uptake was not reached during the course of the experiment; the earlier experiments in which dimethylcatechol concentrations were estimated indicated that this is because the concentration of dimethylphenol remained sufficiently high in these cases to exert a strong inhibitory effect throughout the experiments. At the lowest dimethylphenol concentrations (less than 0·0004M) the rapid phase of oxygen uptake finishes before all the ascorbic acid has been oxidized (which requires 508 μl. O_2); it thus appears that in this concentration range the rate of enzyme inactivation becomes greater with diminishing substrate concentration.

Inhibition of diphenol oxidation by monophenol. The inhibitory effect of 4:5-dimethylphenol on the oxidation of 4:5-dimethylcatechol was demonstrated directly in systems containing ascorbic acid, enzyme and known concentrations of the two substrates. The results of a typical experiment are shown in Fig. 12, where the course of oxygen uptake in the first few minutes in systems containing 10^{-3}M-dimethylcatechol and various concentrations of dimethylphenol are plotted. There is a marked depression of the initial uptake rate with 5×10^{-6}M-monol, i.e. when the ratio of monol to diol is only

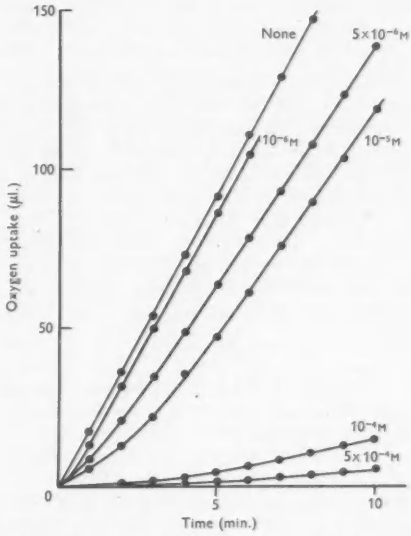

Fig. 12. Effect of 4:5-dimethylphenol on oxidation in the 4:5-dimethylcatechol + ascorbic acid + tyrosinase system; 0·029 mg. enzyme (dry wt.); 4:5-dimethylcatechol concentration, 10^{-3}M; 4:5-dimethylphenol concentrations as stated against each curve.

1:200, and the inhibition is practically complete when this ratio is 1:10. At the lower monol concentrations the inhibition quickly passes off, as may be expected owing to the conversion of monol to diol by the enzyme. A rough quantitative estimate of the extent of the inhibition in this and another similar experiment is given in Table 4. The estimate is based on the oxygen uptake in the first minute; but it should be remembered that with the very low monol concentrations the inhibition may already be passing off even within this short time, on account of diminishing monol concentration.

Table 4. *Inhibition of oxidation of* 4:5-*dimethylcatechol by* 4:5-*dimethylphenol*

Initial concentration (10^{-3} M)				
Diol	Monol	Ratio: monol/diol	Initial O_2 uptake rate (μl./min.)	Inhibition (%)
1·0	Nil	—	18·0	—
	0·001	0·001	13·5	25
	0·005	0·005	9	50
	0·01	0·01	6·5	64
	0·1	0·1	1	94
	0·5	0·5	0·5	?100
0·3	Nil	—	20	—
	0·0012	0·004	8·5	57
	0·005	0·016	5	75
	0·02	0·07	1·5	93

Oxidation of 4:5-*dimethylphenol in absence of ascorbic acid.* In this case, the induction period before maximal oxygen-uptake rate was attained was much longer than in the cases of the other monophenolic substrates under the same conditions. The oxygen uptake in such an experiment is included in Fig. 3. The maximal rate corresponded to a Q_{O_2} of 620, reached after the reaction had proceeded 100 min. This may be compared with the value of 330 found for the isolated primary reaction in the presence of ascorbic acid at a similar dimethylphenol concentration.

DISCUSSION

The behaviour of the reacting system containing monophenol, tyrosinase and ascorbic acid can in each of the cases studied be adequately explained in terms of: (*a*) specific monophenolase activity of the enzyme preparation, maximally effective, in the presence of ascorbic acid, at the moment of addition of enzyme to substrate; (*b*) specific catecholase activity of the preparation; (*c*) progressive inactivation of the enzyme during the reaction; and (*d*) competitive inhibition of catecholase activity by monophenols. The relative magnitude of these factors determines the course of oxidation in each particular case. Comparing the oxidation of tyrosine + ascorbic acid with that of 4:5-dimethylphenol + ascorbic acid, for example, we find that the Q_{O_2} values for the isolated primary oxidations were not very different (230 for 0·0025 M-tyrosine, 180 for 0·005 M-dimethylphenol). The oxygen uptake reached its maximal rate much more rapidly with tyrosine than with dimethylphenol, because dimethylphenol strongly inhibits the oxidation of the dimethylcatechol formed from it in the primary reaction, whereas tyrosine has no such inhibitory effect on DOPA oxidation. Again, the effect of this inhibition in accentuating and prolonging the induction phase of the oxygen-uptake curves is much more striking in the case of dimethylphenol than with phenol or *p*-cresol not only because the inhibition for a given monol/diol concentration ratio is more powerful, but also because the rate of diphenol formation is much slower, the relative monophenolase activities for the substrates phenol/*p*-cresol/dimethylphenol being 1·0/2·6/0·1, whereas the relative catecholase activities with catechol/homocatechol/dimethylcatechol were 1·00/0·83/0·59.

Reference was made in the introduction to the conclusion of other workers that the oxidation of monophenols by tyrosinase is dependent in some way upon the simultaneous oxidation of *o*-diphenol by the enzyme, though not a result of secondary oxidation of monophenol by products of oxidation of diphenol. In the case of each monophenol studied in the present work, however, the rate of the primary oxidation to *o*-diphenol appears to be maximal at the very beginning of the enzyme reaction, and, when an induction period is evident before the maximal oxygen-uptake rate is reached, it is due in the main to the inhibiting effect of monophenol on diphenol oxidation. This leads inevitably to the conclusion that the presence of *o*-diphenol is not in fact necessary for monophenolase activity to be displayed. How then is the induction period observed when monophenol is oxidized in the absence of ascorbic acid to be explained? With 4:5-dimethylphenol as substrate the Q_{O_2} of the primary reaction in the presence of ascorbic acid was found to be of the order of 300 for the enzyme used in this work. In the absence of ascorbic acid, with the same substrate, the total oxygen-uptake rate reached a value corresponding to $Q_{O_2} = 620$, but only after a long induction period during the earlier part of which the oxygen uptake rate was almost zero (Fig. 3). This maximal value of 620 does not conflict with a Q_{O_2} (monophenolase) of about 300, but the fact that the initial oxygen-uptake rate was very much smaller than that appropriate to a Q_{O_2} of 300 points to the conclusion that in the absence of ascorbic acid the monophenolase function of the enzyme was at first relatively quiescent, and that it was activated by *o*-diphenol as the latter slowly accumulated. Ascorbic acid appears to bring about an almost instantaneous activation of monophenolase function and its effect in this respect is not, as other workers have supposed (Nelson & Dawson, 1944), dependent on its favouring *o*-diphenol accu-

mulation. Indeed from the moment of completion of the reaction mixture by addition of enzyme onwards, monophenolase activity diminishes on account of enzyme inactivation, in spite of the progressive increase of *o*-diphenol concentration and oxidation. Ascorbic acid and *o*-diphenols have in common rather strong reducing power and similar reducing groups, and it seems possible that the effect which they have on monophenolase function may be dependent on this property. At any rate the new evidence is against the existence of any specific role for *o*-diphenols in relation to monophenol oxidation.

It is regrettable that the methods available for *o*-diphenol estimation did not permit the studies of *o*-diphenol formation to be carried out over a sufficient range of monophenol concentration to provide reliable activity-substrate concentration curves for the isolated primary oxidation. With each monophenol substrate, monophenolase activity was highest at the highest substrate concentration employed, even when this was as great as 0·1 M, and there was no sign of inhibition such as is observed with the *o*-diphenolic substrates at such high concentrations. The strong competitive inhibitory effect of quite low concentrations of 4:5-dimethylphenol on the catecholase function of the enzyme suggests a much greater affinity of the specific 'catecholase centre' in the enzyme for this monophenol than for the *o*-diphenols or other monophenols used. Were the same centre responsible for monophenol oxidation, one would expect the substrate concentration necessary to saturate the enzyme to be particularly low in the case of dimethylphenol. The few facts available are not in accordance with this expectation. In the case of dimethylphenol the Q_{O_2} (monophenolase) was 330 at 0·02 M-substrate, but only 180 at 0·005 M-substrate, whilst with *p*-cresol a value of 7200 at 0·01 M had fallen only to 5000 at 0·001 M, suggesting that the affinity for dimethylphenol was appreciably less than for *p*-cresol. Thus it appears probable that the activities of the enzyme or enzyme complex in respect of monophenol and *o*-diphenol are located at different centres.

The optimal concentrations of the *o*-diphenolic substrates were 0·004 M for catechol, 0·006 M for homocatechol and 0·00035 M for 4:5-dimethylcatechol. Two of these values agree reasonably well with the figures given by Nelson & Dawson (1944), 0·002 and 0·0007 M for catechol and dimethylcatechol respectively. For homocatechol these authors give 0·0006 M as the optimal concentration and there appears to be no obvious explanation of the discrepancy. It would be unwise to assume that these differences in optimal concentration reflect differences in enzyme-substrate affinities, in the absence of knowledge of other factors which determine the shapes of the activity-substrate concentration curves, and in particular of the reason for the falling off in activity at high substrate concentrations, which is especially marked in the case of dimethylcatechol. The relative activity towards the three *o*-diphenolic substrates, at the optimal concentration of each, of 1·00:0·83:0·59, does not differ greatly from the ratio of 1·00 : 0·88 : 0·74 quoted by Nelson & Dawson (1944).

So far as the experiments which have been described show, the monophenolase and catecholase activities of tyrosinase appear to be functionally independent, except as regards the inactivation of the enzyme during the reaction. In the treatment of the data obtained in the monophenol oxidation experiments, the rate of formation of *o*-diphenol was taken as a measure of the amount of enzyme still in the active condition, in those experiments in which it was considered that the decline in this rate could not be attributed to the diminution in monophenol concentration. Catecholase activity, assessed in terms of oxidation rate of ascorbic acid, was then related to the amount of residual active enzyme determined in this way. The Q_{O_2} (DOPA) values thus calculated from the data of the tyrosine oxidation experiment were found to agree very well with those obtained by direct manometric studies of the oxidation of DOPA itself (Fig. 2), and this appears to justify the calculation; but in the calculation the amount of residual active enzyme, to which the DOPA oxidation was attributed, was judged by the extent of residual monophenolase activity. This suggests that the process of inactivation affects equally both the catecholase and monophenolase functions, and, therefore, that these are properties of one and the same enzyme complex. The extent to which the data relating to the oxidation of the other monophenols conform with this view is not readily judged, because of the additional complicating factor of inhibition of *o*-diphenol oxidation by the monophenol present.

Nearly all previous experimental work on the oxidation of monophenols by tyrosinase has been carried out with the initially simpler system containing only monophenol, enzyme and buffer. The quantitative study of *o*-diphenol formation in such systems presents obvious difficulties on account of the pigment developed, but it is hoped that such studies may soon be made. It is by no means certain, however, that the behaviour of the enzyme in the absence of ascorbic acid is of greater biological significance than its behaviour in the presence of this substance. Of the two sources of tyrosinase which have been chiefly used, the mushroom is stated to contain no ascorbic acid (Medical Research Council, 1945), but the potato may contain up to 30 mg./100 g. Several authors have suggested that the *o*-diphenol-tyrosinase system may play a part in the economy of plant tissues, by forming the terminal link in a respiratory chain (Boswell & Whiting, 1938;

Baker & Nelson, 1943). According to their view the *o*-quinone formed in the presence of oxygen by the enzyme abstracts hydrogen from some unspecified hydrogen donator and is consequently reconverted to *o*-diphenol. In the potato one would expect ascorbic acid to be a powerful competitor for this quinone, unless it is in some way separated spatially from the enzyme in the tissue cells. The monophenolase function appears to have been regarded by the proponents of this theory merely as a mechanism for providing the *o*-diphenol necessary for such a respiratory function. Robinson & Nelson (1944) formed the opinion that in the presence of tyrosinase only a trace of DOPA sufficient for the above purpose accumulated, and suggested, to explain this, that the oxidation of tyrosine was strongly inhibited by DOPA. No direct experimental evidence in support of these speculations was given, however, and the evidence now presented has shown that considerably more than traces of DOPA may accumulate. There was, it is true, a falling off in the rate of DOPA formation during the enzyme reaction, although the tyrosine concentration remained constant, but this can be adequately accounted for by enzyme inactivation. The possibility that the monophenolase function of tyrosinase may also be important for syntheses of substances other than the substrate for the catecholase function ought still to be borne in mind.

SUMMARY

1. Colorimetric methods for the estimation of *o*-diphenol in enzymic reaction mixtures containing ascorbic acid and the corresponding monophenol are described.

2. The results of studies of the course of oxygen uptake, *o*-diphenol formation and disappearance of ascorbic acid during the oxidation by tyrosinase, in the presence of ascorbic acid, of tyrosine, phenol, *p*-cresol and 4:5-dimethylphenol (3:4-dimethylphenol) are reported.

3. The primary oxidation of monophenol to *o*-diphenol by tyrosinase in the presence of ascorbic acid was found to proceed at maximal rate at the moment of addition of enzyme to substrate. There was no induction period such as is observed in the absence of ascorbic acid. This leads to the conclusion that *o*-diphenol plays no specific part in facilitating the oxidation of monophenols by tyrosinase.

4. The behaviour of the reacting system containing initially monophenol, tyrosinase and ascorbic acid can be adequately explained in terms of (*a*) specific monophenolase activity of the enzyme preparation; (*b*) specific catecholase activity of the enzyme preparation; (*c*) progressive inactivation of the enzyme during the reaction; (*d*) competitive inhibition of catecholase activity by monophenols.

5. The bearing of the results on current views of the nature and action of tyrosinase is discussed. It is suggested that they support the view that tyrosinase is a single enzyme or enzyme complex having independent centres associated specifically with its monophenolase and with its catecholase functions.

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Polysaccharides Synthesized by Aerobic Mesophilic Spore-forming Bacteria

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In the classification proposed by Smith, Gordon & Clark (1946) the genus *Bacillus* has been divided into three groups depending principally on morphological differences in the size and shape of the spores and the shape of the sporangia. Their classification and nomenclature has been used throughout this paper.

The bacilli of group I are known to synthesize fructosans from sucrose; those obtained from *B. subtilis*, *B. megatherium* and *B. pumilus* (*mesentericus*) have been intensively studied (Harrison, Tarr & Hibbert, 1930; Challinor, Haworth & Hirst, 1934; Lyne, Peat & Stacey, 1940; Evans & Hibbert, 1946) and all have been shown to be levans of the same general structure containing D-fructose as the sole constituent sugar.

The same attention has not been paid to the polysaccharides produced by the bacilli of group II. *B. polymyxa* has been shown to produce a levan from sucrose, and an unidentified polysaccharide which is not a levan from other sugars (Hestrin, Avineri-Shapiro & Aschner, 1943). Kleczkowska, Norman & Snieszko (1940) isolated a new capsulated bacillus from cultivated soil which they named *B. krzemieniewski*. In some strains well-defined capsules were present, while in others the mucilage was dispersed throughout the medium. Kleczkowski & Wierzchowski (1940) reported the separation of the capsular polysaccharide, and solely on the optical rotation of the total hydrolysate and the isolation of mannose phenylhydrazone, deduced that the polysaccharide was a mannan composed of L-mannose units. The sugar L-mannose has not been known to occur previously, and it has been commented (Evans & Hibbert, 1946) that such a result requires confirmation. *B. krzemieniewski* is now considered by Smith *et al.* (1946), pending the study and isolation of more strains, as a mucoid stage of growth of *B. circulans*. It is classified as *B. circulans* 760 in their collection. Group III bacilli do not appear to produce exocellular polysaccharides.

It was not our intention to make an intensive structural analysis of any of the polysaccharides produced by the bacilli, but rather, by studying the nature of the constituent sugars, to discover if any correlation exists between the groups of the above and the type of polysaccharide synthesized in presence of various sugars.

EXPERIMENTAL

Cultures. We are grateful to Dr Ruth E. Gordon, the Curator of the American Type Culture Collection, for her kindness in sending us the following strains of polysaccharide-producing bacilli from their collection.

Group I

	Strain no.		Strain no.
B. megatherium	697	*B. subtilis*	1248
B. pumilus	355	*B. cereus*	793

Group II

B. polymyxa	354	*B. circulans*	294
B. brevis	799	*B. circulans*	295
B. alvei	683	*B. circulans* *B. macerans* intermediate	396

A culture of *B. circulans* strain no. 760 (*B. krzemieniewski*) was also sent. The culture, as received, was a mixture of the smooth and mucoid stages. These were separated on nutrient agar and designated for convenience *Km* (mucoid stage) and *Ks* (smooth stage). Both were tested for gum production on medium II of Cooper & Preston (1937). *Km* produced the typical colonies depicted by Kleczkowska *et al.* (1940), resembling glass beads in appearance, firm but resilient, and adhering strongly to the medium. *Ks* also produced gummy colonies on this medium, but the colonies were not firm, and the growth soon coalesced to form a gummy mass on the surface of the plate. The mucoid stage was unstable and slowly reverted to the smooth stage either on frequent subculture on Cooper & Preston's medium or after prolonged incubation or storage on this medium. It seems probable that *Km*, which is capsulated, is the same as strain A of Kleczkowska *et al.* (1940), while *Ks* corresponds to their strain B. These American cultures, Dr Gordon informs us, have been in storage for 6–10 years. In addition to the above, the following cultures of *B. polymyxa* were available for study: *B. polymyxa* N.C.T.C., nos. 1380, 4745, 4744, and a strain of *B. polymyxa* isolated in Prof. Kluyver's laboratory (obtained through the kindness of Dr Swaby of Rothamsted). Culture no. 4744 was that used by Hestrin *et al.* (1943).

Polysaccharide production. For the preparation of sufficient polysaccharide for analysis of the constituent sugars, cultures of each organism were grown on the surface of agar in four 9 cm. Petri dishes. The dishes, each containing 15–20 ml. of Copper & Preston's (1937) basic medium II with 2% of the various sugars, sterilized separately, were heavily inoculated and incubated at 25° for 2–7 days, depending on the organism. The surface growth was removed by means of

a sterile bent glass rod after the addition of a minimal quantity of sterile distilled water to facilitate harvesting. With *Km*, because of the toughness of the colonies and their adhesion to the agar surface, it was often necessary to leave the distilled water in contact with the surface growth for 24 hr. in the incubator. This was usually sufficient to detach the colonies, which could then be removed without any danger of scraping up pieces of agar. Frequent tests subsequently showed that with reasonable care no contamination from the agar would be encountered. Polysaccharide synthesis by each organism was tested on sucrose, D-fructose, D-glucose, D-galactose, and L-arabinose. With each bacillus, polysaccharides containing identical sugar constituents were produced from the last four sugars, although in different yields. For brevity, description will thus be limited to the polysaccharides produced on sucrose and monosaccharides.

Partial purification of polysaccharides. The growth, washed off with distilled water from four Petri dishes, was precipitated by dropping into 4 vol. of ethanol containing 2% glacial acetic acid and stirring vigorously. After standing for 1 hr. the precipitate was filtered on a sintered-glass funnel and washed with ethanol of increasing strength, and then with ether. When dried in this manner the crude gums were generally in the form of fine white powders which could be redissolved in distilled water. Impurities could be spun down, and the polysaccharide reprecipitated with ethanol-acetic acid and dried as before. Where the mucilages were not readily dissolved in water, it was sometimes necessary to redissolve in 0·1 N-NaOH. The products generally contained a considerable quantity of nitrogenous material derived from the bacteria. While the yield of this gum varied with the bacillus and the substrate sugar, in general *c.* 50–150 mg. were obtained from the four Petri dishes.

Hydrolysis of the polysaccharides. Two hydrolyses were carried out on each product: (*a*) *c.* 20 mg. gum were hydrolyzed with 0·5 ml. 0·1 N-H_2SO_4 for 30 min. in a sealed ampoule in a water bath at 100°; (*b*) *c.* 20 mg. gum were hydrolyzed with 0·5 ml. N-H_2SO_4 for 4 hr. as above. After the completion, the seals were broken and the hydrolysate neutralized to Congo red with solid $BaCO_3$. The Ba salts were then centrifuged down, still in the ampoule, and the supernatant liquid used for determination of sugars. The short hydrolysis in 0·1 N-H_2SO_4 serves to show the presence of furanose sugars such as D-fructose which would be almost totally destroyed in the stronger hydrolysis. When fructose was found to be present, the remainder of the gum was generally hydrolyzed with 0·5% oxalic acid for 1 hr. on a water bath and the fructose-free residue, if present, was precipitated with ethanol, washed and dried as before. The residue could then be hydrolyzed and analyzed without interference from fructose and its decomposition products.

Identification of constituent sugars. The sugars present were identified by the paper chromatographic technique (Partridge, 1946, 1948; Forsyth, 1948). From the R_F values of the constituent sugars using *n*-butanol, phenol, and *s*-collidine as solvents, by comparison with the position of standard sugars on the papers and by spraying with resorcinol and naphthoresorcinol (Forsyth, 1948), it was possible to identify all the sugars which were encountered. Uronic acids were detected both by the naphthoresorcinol test on the gum itself, and by spraying chromatograms of the hydrolysates with the reagent. In some cases quantitative estimations of sugars were made, after extraction from chromatograms, by the method of Flood, Hirst & Jones (1947).

RESULTS

Polysaccharides of Bacillus cereus 793, Bacillus subtilis 1248, Bacillus pumilus 355

When grown on sucrose these bacilli produced copious yields of gum, but no synthesis took place on a monosaccharide substrate. On hydrolysis with 0·1 N-sulphuric acid or 0·5% oxalic acid, no appreciable residue of ethanol-insoluble material remained, and only one spot was obtained on paper chromatograms, which was readily identified as fructose from its R_F values (*n*-butanol 0·10; phenol 0·51; *s*-collidine 0·42) and its colour reaction on spraying with naphthoresorcinol. That D-fructose was the reducing sugar present was readily confirmed in each case by measuring the optical rotation and the reducing sugar value of a hydrolysate of the mother sample of the gum.

Polysaccharide of Bacillus megatherium 697

This bacillus produced polysaccharides on both the sucrose and monosaccharide media. The gum from the sucrose substrate, on hydrolysis with the 0·1 N-sulphuric acid gave only D-fructose, identified as before. In the more concentrated N-sulphuric acid, however, the fructose was mainly destroyed giving only a faint spot, whereas another sugar was present which proved to be glucose. After hydrolysis in oxalic acid *c.* 40% of ethanol-insoluble residue still remained, which on further hydrolysis with N-sulphuric acid gave a hydrolysate free from fructose, but containing the other sugar as well as uronide material. This sugar was shown by spraying with resorcinol to be an aldose and was readily identified as glucose from its R_F values (*n*-butanol 0·07; phenol 0·39; *s*-collidine 0·39). This was confirmed by the preparation of glucosazone from the hydrolysate of the fructose-free residual polysaccharide. The gum from the monosaccharide media contained no fructose, giving only glucose and uronic material on hydrolysis. The presence of uronides was confirmed by the naphthoresorcinol test. It is probable that the gum produced on sucrose media is a mixture of levan and the glucose-uronic polysaccharide, which is synthesized alone on the monosaccharide media.

When grown on the liquid media of Tarr & Hibbert (1931) with the addition of 2% yeast extract and with sucrose as substrate sugar, only levan was produced, no glucose being detected, and the gum was completely hydrolyzed with oxalic acid. This phenomenon has been more closely investigated with *B. polymyxa*.

Polysaccharide of Bacillus polymyxa

All the strains of *B. polymyxa* investigated gave the same results. When grown on monosaccharide media a fructose-free polysaccharide was obtained,

which on hydrolysis with N-sulphuric acid, but not with 0·1 N-sulphuric acid, gave a hydrolysate shown by chromatography to contain two sugars. One of these was glucose and the other was identified as mannose from its R_F factors (*n*-butanol 0·10; phenol 0·45; *s*-collidine 0·46) and resorcinol spray, which showed it to be an aldose. This was confirmed by the preparation of mannose phenylhydrazone from a hydrolysate of the remainder of the gum. Uronides were also present.

When grown on sucrose medium a gum was obtained, which on hydrolysis with dilute acid gave fructose, identified as before. On hydrolysis with stronger acid a more complex sugar mixture was formed. With *n*-butanol two spots were present with R_F factors corresponding to glucose and fructose, but with phenol and *s*-collidine three spots were apparent. Apart from the glucose and fructose, mannose was also identified. Mannose and fructose have the same R_F factor in butanol. After removal of the fructose with oxalic acid, the residual polysaccharide gave the same sugars on hydrolysis as were produced from the polysaccharide formed on the monosaccharide substrate. As in the case of *B. megatherium*, a much larger proportion of levan was formed when *B. polymyxa* was grown on Tarr & Hibbert's liquid medium.

Dependence of levan production on culture media

As we have seen, both *B. megatherium* and *B. polymyxa* produce, from sucrose on Tarr & Hibbert's (1931) liquid medium, mainly levan, whereas on Cooper and Preston's (1937) solid media a considerable proportion of other polysaccharides, which are the sole products from a monosaccharide substrate, is produced. This difference may be due to one, or more than one, of the following factors: (1) aeration differences between solid and liquid culture; (2) differences in sucrose concentration (2 % in Copper & Preston's media and 10 % in that of Tarr & Hibbert); (3) intrinsic differences in the basic media such as phosphate concentration.

Accordingly, *B. polymyxa* was grown under a variety of conditions, and levan estimated as reducing sugar after hydrolysis of the gums with oxalic acid. The residual ethanol-insoluble polysaccharide was also estimated by isolation and weighing. The results are shown in Table 1. The residual polysaccharide, even when only present in traces, gave glucose and mannose on hydrolysis. It is apparent from Table 1 that the aeration factor plays no part in levan production. Indeed a similar mixture of polysaccharides is produced when *B. polymyxa* is grown anaerobically. High concentrations of both sucrose and phosphate appear to be necessary for optimal levan production.

Table 1. *Levan production by* Bacillus polymyxa

Basic media	Type of culture	Sucrose (%)	PO_4 (g./l.)	Levan (% of total gum)	Residual gum as % of total gum
Cooper & Preston (1937)	Liquid	2	0·11	44	43
	Solid	2	0·11	39	39
	Solid	10	0·11	37	41
	Solid	10	0·53	69	13
Tarr & Hibbert (1931)	Liquid	10	0·53	86	7
	Solid	10	0·53	70	4
	Solid	2	0·53	37	40
	Solid	10	0·11	57	25

Polysaccharides of Bacillus brevis 799, Bacillus alvei 683

These strains produced polysaccharides containing the same sugars on both sucrose and monosaccharide substrates. No levan was formed. On hydrolysis with N-sulphuric acid, glucose and a uronide fraction were the only sugars identified.

Polysaccharides of Bacillus circulans 294, Bacillus circulans, Bacillus macerans *intermediate* 396

These bacilli, like the last two and all subsequently to be discussed, produced polysaccharides containing the same sugars on both sucrose and monosaccharide substrates and did not synthesize levan. Hydrolysis of the gum with N-sulphuric acid gave two sugars which were identified as before from their R_F factors and resorcinol spray as glucose and mannose. Uronides were also present.

Polysaccharide of Bacillus circulans 295

Hydrolysis of the gum produced by this bacillus with N-sulphuric acid gave a mixture of three sugars as well as uronic residues. Two of the sugars were readily identified as before as glucose and mannose. The third sugar was shown to be a pentose by resorcinol spray and was identified as xylose from its R_F factors (*n*-butanol 0·13; phenol 0·44; *s*-collidine 0·50). Comparison was made on chromatograms with the other three pentoses, arabinose, ribose, lyxose, in order to confirm that the sugar was xylose. The presence of xylose was further confirmed by the preparation of the dimethyl acetal of dibenzylidene xylose (Breddy & Jones, 1945; Wise & Ratcliff, 1947) from the sugar syrup prepared from a hydrolysate of the remainder of the gum.

Polysaccharides of Bacillus circulans 760 (Bacillus krzemieniewski)

These have been most carefully studied since it has been claimed (Kleczkowski & Wierzchowski, 1940) that a mannan composed of L-mannose units is the main polysaccharide synthesized.

Ks (*smooth form*). The strain was grown on a range of sugars and the crude gum isolated as for the other bacilli previously discussed. A rough estimate of the production of polysaccharide by the strain on different sugars may be gained from a comparison of the yields of this partly purified product shown in Table 2. The gums contained no levan, and on hydrolysis gave glucose and mannose, identified chromatographically as before.

Table 2. *Yields of gum by* Bacillus circulans 760 (*smooth form*) *on different sugars*

(Growth at 25° for 7 days)

	mg./plate		mg./plate
Sucrose	108	D-Galactose	24
Mannitol	62	Lactose	24
D-Glucose	58	L-Arabinose	14
D-Fructose	46	L-Rhamnose	4

Km (*mucoid form*). Similar analysis was made on the polysaccharide synthesized by the well-capsulated form of the organism and the same constituent sugars were found to be present. To exclude the possibility that this polysaccharide, which is of a more complex nature than a simple mannan, is synthesized because of different culture conditions, both *Ks* and *Km* were grown on the media used and the conditions prescribed by Kleczkowski & Wierzchowski (1940). Again both glucose and mannose were detected in the hydrolysates.

For further investigation larger amounts of gum were prepared from *Ks*, and further purified in a similar manner to that described by Kleczkowski & Wierzchowski (1940): 5 g. of the gum were heated under reflux with 300 ml. N-KOH for 3 hr. on a water bath at 100°, and the hot solution filtered through glass wool and then centrifuged. On cooling a very viscous gel formed. The alkaline solution was poured into 4 vol. of ethanol containing acetic acid, washed with ethanol, then ether, and dried. When purified by electrodialysis, as described by Kleczkowski & Wierzchowski, a low yield of purified product was obtained. Nevertheless, this method was used with a portion of gum to obtain sufficient material for sugar analysis. On hydrolysis of the electrodialyzed polysaccharide, glucose and mannose were formed as before. The bulk of the material was further purified in good yield by forming the copper complex by the addition of $CuSO_4$ to an alkaline solution of the gum. The complex was washed with hot water, and then decomposed with ethanol containing HCl and washed free from Cu^{++} and Cl^- ions with ethanol. After drying with ether, the regenerated gum was redissolved in a small volume of 0·1 N-NaOH and precipitated by a large excess of glacial acetic acid. After drying with ethanol and ether the product (1·45 g.) was a fine white powder, difficultly soluble in water to give an opalescent, very viscous solution. It was readily soluble in dilute alkali and was almost free from ash and N. After electrodialysis it gave an equivalent weight of 598 on titration with 0·1 N-NaOH using phenolphthalein as indicator. The equivalent weight was not significantly altered on fractional precipitation of the polysaccharide with ethanol. Determination of uronic anhydride by CO_2 evolution on refluxing with 12% HCl gave a value of 28·9% (calculated from equivalent weight, 29·8%). This uronic acid would give rise to *c.* 6·3% furfural (found, 6·1%) which confirms the absence of pentoses. Glucose, mannose, and a uronic acid were again shown to be present by paper chromatography.

The sugars on the paper chromatograms were determined by the method of Flood *et al.* (1947): glucose/mannose ratio (Exp. 1) 1·53; (Exp. 2) 1·45. Using a known weight of xylose as an internal standard, the actual percentages of glucosan and mannan were determined (Table 3). Mannose was also estimated as the phenylhydrazone by the method of Jones, Hirst & Woods (1947).

Table 3. *Composition of purified gum from mucoid form of* Bacillus circulans 760

	Glucosan (%)	Mannan (%)	Uronic anhydride (%)	Equiv. wt.
Theoretical	41·8	27·9	30·3	581
Found	40·5	25·6 25·8*	28·9	598

* As phenylhydrazone.

Hydrolyses with 0·2% oxalic acid for 4 hr. or with 0·1 N-H_2SO_4 for 30 min. at 90° were not effective in splitting off reducing sugars, suggesting the absence of furanose sugars: 1·0 g. polysaccharide was refluxed with 50 ml. N-H_2SO_4 for 3 hr., and the change in rotation determined (1 hr., $[\alpha]_D^{20°}$ +29·5°; 2 hr., +33·3°; 3 hr., +33·3°). While this rotation is of the right order for a mixture of D-uronic acids, D-glucose and (?)-mannose, such as are produced, it is in direct contrast to that of Kleczkowski & Wierzchowski, who obtained a negative rotation. The hydrolysate was neutralized with $BaCO_3$, centrifuged and concentrated. On addition of methanol no barium aldobionate fraction separated. The solution was concentrated to a syrup and taken up in 10 ml. water and mannose phenylhydrazone prepared, m.p. 192°. The hydrazone was then decomposed by boiling in water with a slight excess of benzaldehyde, and, after filtration and removal of the excess benzaldehyde with ether, the regenerated mannose was shown by optical rotation, $[\alpha]_D^{20°} = +14°$, to be D-mannose. That mannose was the sugar regenerated was confirmed by paper chromatography.

DISCUSSION

The results obtained are tabulated in Table 4. It is apparent that levan formation is confined to group I bacilli, with the exception of *Bacillus polymyxa*, and the synthesis of other polysaccharides to group II bacilli, with the exception of *B. megatherium*. *B. polymyxa* and *B. megatherium* might be considered as intermediate since they produce varying proportions of both levan and other polysaccharides from sucrose depending on the culture conditions. *B. circulans* strains produce polysaccharides containing glucose, mannose and a uronic acid. The nature of the polysaccharide is independent of the substrate sugar. It is, of course, by no means proved that these complex polysaccharides are not merely

mixtures of simple dextrans, mannans and polyuronic acids, but attempts to fractionate them by fractional ethanol precipitation have proved unsuccessful. We have not been able to confirm Kleczkowski & Wierzchowski's (1940) claim that *B. krzemieniewski* produces a mannan composed of L-mannose units. The strains available synthesized complex polymers of glucose, mannose and uronic acid in the approximate ratio 3:2:2. The mannose was D-mannose and not the unnatural L-form. *B. circulans* 295 gum is unique in that it contains the pentose, xylose.

Table 4. *Constituent sugars of the mucilaginous polysaccharides synthesized by the genus* Bacillus

Bacilli			Levan formation on sucrose	Other polysaccharides synthesized on sucrose and monosaccharides
Group	Species			
I	*cereus*	793	+	—
	pumilus	355		
	subtilis	1248		
	megatherium	697	+	Glucose-uronic
II	*polymyxa*	354	+	Glucose-mannose-uronic
	circulans	760		
	circulans	294	–	
	circulans-macerans	396		
	brevis	799	–	Glucose-uronic
	alvei	683		
	circulans	295	–	Glucose-mannose-xylose-uronic

It may be of some interest that when a crude ethanol precipitate of a culture of these bacilli is hydrolyzed, ribose, presumably from the nucleic acids, is readily identified among the polysaccharide sugars.

SUMMARY

1. The constituent sugars of polysaccharides synthesized by the genus *Bacillus* from sucrose and monosaccharide sugars have been identified. Strains of *B. cereus*, *B. pumilus*, *B. subtilis*, *B. megatherium*, *B. polymyxa*, *B. circulans*, *B. circulans-macerans*, *B. brevis*, and *B. alvei* have been studied.

2. There appears to be a relation between type of polysaccharide synthesized and the group classification as proposed by Smith *et al.* (1946). Levan synthesis from sucrose is confined to group I bacilli and *B. polymyxa*. On the other hand, the synthesis of other polysaccharides from sucrose and monosaccharides is confined to group II bacilli and *B. megatherium*.

3. *B. polymyxa* and *B. megatherium* are intermediate in that they synthesize both levan and other polysaccharides from sucrose depending on the culture medium.

4. Group II bacilli synthesize from a wide variety of sugars polysaccharides containing (*a*) glucose and a uronic acid; (*b*) glucose, mannose, and a uronic acid; (*c*) glucose, mannose, xylose, and a uronic acid. The type of polysaccharide is characteristic of the bacillus and not of the substrate sugar.

5. We have not been able to confirm Kleczkowski & Wierzchowski's (1940) claim that *B. krzemieniewski* produces a mannan composed of L-mannose units. The strains available synthesized a complex polymer of glucose (3 parts), mannose (2 parts) and uronic acid (2 parts). The mannose was the 'natural' D-mannose.

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The Pathway of the Adaptive Fermentation of Galactose by Yeast

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Certain yeasts can ferment galactose by the formation of an adaptive 'galactozymase' when placed in aqueous solutions of galactose and in the apparent absence of cell division (Stephenson & Yudkin, 1936; Spiegelman, 1945). Though much work has been done on the mechanism of this adaptation (Spiegelman, 1947) little knowledge has so far been obtained concerning the actual enzymic changes involved or of the intermediates formed.

Kosterlitz (1943*b*), who has reviewed the information available, showed that a maceration juice prepared from galactose-adapted yeast would ferment both α-glucose-1-phosphate and α-galactose-1-phosphate in a similar manner; he suggested that α-galactose-1-phosphate played the same role in galactose fermentation as did α-glucose-1-phosphate and glucose-6-phosphate in glucose fermentation. Working with dialyzed yeast extracts, Kosterlitz showed that 27% of the α-galactose-1-phosphate added was converted into a difficultly hydrolyzable ester. In the same time, about 90% of α-glucose-1-phosphate was converted to glucose-6-phosphate. To explain these results, he suggested that at least two new enzymes were involved in galactose fermentation: one converting galactose to α-galactose-1-phosphate, and a second converting this ester into α-glucose-1-phosphate which is then transformed into the Robison ester.

The work presented in this paper describes the preparation of an extract from galactose-adapted yeast and the formation of an easily hydrolyzable phosphoric ester from galactose and adenosinetriphosphate in the presence of this extract. This ester has been identified as α-galactose-1-phosphate. Some evidence is also presented in favour of the view that galactose-1-phosphate is further transformed into glucose-6-phosphate.

EXPERIMENTAL

Organisms, growth media and preparation of extracts. The work was carried out with a strain of Dutch top yeast (Cat. no. 174) obtained from the Carlsberg Laboratories. The yeast was grown in Roux bottles, each of which contained 150 ml. of the following medium: 0·1% $NH_4H_2PO_4$; 0·1% $(NH_4)_2HPO_4$; 0·2% KH_2PO_4; 0·2% Difco Yeast Extract; 2% galactose; 1% of a trace-element solution. The trace-element solution contained, per litre: $MgSO_4.7H_2O$, 20·0 g.; NaCl, 1·0 g.; $FeSO_4.7H_2O$, 0·5 g.; $ZnSO_4.7H_2O$, 0·5 g.; $MnSO_4.3H_2O$, 0·5 g.; $CuSO_4.5H_2O$, 0·05 g.; 0·1 N-H_2SO_4, 10 ml.

The medium was sterilized and inoculated from a 24 hr. culture of the yeast in the above medium at 25°. After 36 hr. at 25° the yeast was harvested, washed on the centrifuge with 0·05 M-NaH_2PO_4, and made into a thick suspension in a solution containing 0·1% galactose and 0·01% neutralized Na_2S. The suspension was then shaken with glass beads for 15 min. on a Mickle disintegrator (Mickle, 1948). It was found necessary to carry out the disintegration under anaerobic conditions in order to obtain fully active extracts: such conditions were obtained by the addition of 0·01% neutralized Na_2S to the suspension, and by filling the gas space in the vessels of the disintegrator with H_2. The glass beads were filtered off on a coarse sintered-glass filter funnel, and the filtrate centrifuged at 3000 r.p.m. for 15 min. The cell-free supernatant fluid was used for the experiments.

Reagents and methods. The adenosinetriphosphate (ATP) used was made from a commercial preparation of the barium salt supplied by Boots Ltd. This was purified by dissolving it in 0·2 N-HNO_3 until the solution was just acid to Congo red, and then by following the final stages of the preparation described by Needham (1942). Galactose-6-phosphate was prepared according to the method of Levene & Raymond (1931), and α-glucose-1-phosphate by the method of Hanes (1940). Glucose and galactose solutions were prepared from the pure bacteriological sugars supplied by Kerfoots Ltd.

Acid production. The phosphorylation of hexose by ATP in the 1 or 6 position results in an increase in acidity of the reaction mixture. Phosphorylation can therefore be followed manometrically by measuring the CO_2 evolved when the reaction is carried out in a bicarbonate-CO_2 buffer system (Colowick & Kalckar, 1943). Warburg manometers were used for this purpose.

Reducing sugar was estimated by the method of Somogyi (1945). Samples were first deproteinized by means of $ZnSO_4$ and $Ba(OH)_2$, which also removes any phosphorylated hexose esters. Fructose was determined by the method of Roe (1934), and pentose by the method of Meijbaum (1939). The distribution of P in the various fractions was determined by estimation of orthophosphate by the Fiske & Subbarow method (1925), as follows: direct estimation, $P_{0\,min.}$ = ortho-P; after wet ashing = total P; after hydrolysis in N-HCl at 100° for 7 min., $P_{7\,min.}$; by calculation, $P_{7\,min.} - P_{0\,min.}$ = acid-labile P; total $P - P_{7\,min.}$ = acid-stable P.

Separation of the Ba salts of the phosphoric esters formed during the reaction was carried out according to the procedure of Umbreit, Burris & Stauffer (1945).

Differential analysis of 'glucose' and 'galactose' by fermentation was carried out by a modification of the method of Harding & Grant (1931–2). Dutch top yeast was used in

place of baker's yeast, and the disappearance of sugar was followed manometrically by the anaerobic CO_2 evolution. When this was complete, the residual sugar was measured by the method of Somogyi (1945).

RESULTS

Preliminary experiments with the crude extract. Neither glucose nor galactose was fermented by the crude extract, even after the addition of boiled yeast extract, hexosediphosphate, acetaldehyde and ATP, singly or in combination; hexosediphosphate itself was not fermented. The ability of the extract to phosphorylate glucose or galactose in the presence of ATP was tested by following the increase in acidity in bicarbonate buffer, and the results were checked at the end of the reaction by measurement of the free sugar disappearing. The results are shown in Table 1.

Table 1. *Effect of adenosinetriphosphate on the increase in acidity* (CO_2 *output*) *and sugar disappearance on addition of glucose and galactose to extracts prepared from normal and galactose-adapted Dutch top yeast*

(The manometers contained—Main chamber: extract, 0·6 ml.; 0·5 M-NaF, 0·2 ml.; 0·12 M-$NaHCO_3$, 0·5 ml.; 0·1 M-$MgCl_2$, 0·2 ml. Side bulb: 0·12 M-$NaHCO_3$, 0·3 ml.; and, where added, glucose or galactose (20 mg./ml.) 0·2 ml.; ATP (2·5 mg. $P_{7\ min.}$/ml.), 0·4 ml. Gas phase: $N_2 + 5\%$ CO_2; temp. 25°. Incubation time: 30 min., after which the free sugar was measured on a 1 ml. sample.)

	Crude extract		Purified extract	
	Normal	Galactose adapted	Normal	Galactose adapted
	CO_2 output (μmol.)			
Control	0·18	0·18	0·09	0·09
Control + ATP	7·06	7·59	2·05	1·61
Glucose	0·80	0·98	0·09	0·18
Glucose + ATP	10·52	11·68	8·72	8·39
Galactose	0·01	0·00	0·00	0·09
Galactose + ATP	6·97	12·91	2·18	8·35
	Sugar disappearance (μmol.)			
Glucose	0·00	0·56	0·00	0·00
Glucose + ATP	8·78	9·12	12·92	14·21
Galactose	0·00	0·00	0·00	0·00
Galactose + ATP	0·22	10·00	0·00	13·98

There is no significant CO_2 output or sugar disappearance in the absence of ATP, whereas in its presence there is a considerable CO_2 production in the control, which is significantly increased in presence of glucose or galactose in the case of the extract prepared from galactose-adapted yeast. In the case of the extract from yeast grown in glucose there is an increased CO_2 production over the control only when glucose is added. The increased CO_2 production after the addition of glucose or galactose can be correlated with the disappearance of sugar in both instances. The large CO_2 output in the control was undesirable, and to minimize this it was necessary to effect some purification of the enzymes concerned.

Purification of the extract. Attempts to remove interfering sugars by dialysis resulted in inactivation of the enzyme system attacking galactose. Even dialysis overnight at 0° against an aqueous solution containing 1% galactose and 0·1% neutralized Na_2S resulted in a complete loss of activity, which could not be restored by the addition of cysteine or of a boiled extract from either normal or galactose-adapted yeast, singly or together. Hexokinase was not inactivated by this dialysis.

The extract was, therefore, fractionated by precipitation, and the following procedure was ultimately adopted. The extract was cooled to 0° and the pH brought to 4·4 by the addition of M-acetate buffer. The precipitate was separated on the centrifuge and discarded. The supernatant fluid was adjusted to pH 7·5 with 0·1 N-NaOH and solid $(NH_4)_2SO_4$ added to bring the final concentration to 75% saturation. After centrifuging, the precipitate was dissolved in a small volume of distilled water and used for the following experiments.

The CO_2 output in absence of sugar was largely eliminated by the purification, as can be seen from Table 1. The increased CO_2 production, after the addition of glucose or galactose, can again be correlated with the disappearance of sugar.

Activation of the enzyme phosphorylating galactose. During the purification process the enzyme lost some activity, which was largely restored by the addition of Mg^{++}, a further increase in activity resulting on the addition of cysteine (Fig. 1). Thus, this system resembles all known phosphokinases in requiring Mg^{++}, and in being activated by thiol compounds.

The presence of phosphoglucomutase and phosphohexose-isomerase in the extract. The presence of these enzymes in the extract was tested as follows. Manometers were set up containing—Main chamber: glucose-1-phosphate (4·0 mg. $P_{7\ min.}$/ml.), 0·7 ml.; 0·5 M-NaF, 0·3 ml.; 0·12 M-$NaHCO_3$, 0·4 ml.; 0·1 M-$MgCl_2$, 0·1 ml.; 0·25 M-cysteine, 0·1 ml. Side bulb: 0·1 M-$NaHCO_3$, 0·25 ml.; extract, 0·75 ml. (boiled extract, 0·75 ml. in control).

The manometers were filled with a CO_2/N_2 mixture containing 5% CO_2, and the contents of the side bulbs tipped into the main cup after equilibration. After shaking for 90 min., 2 ml. of the contents were taken for analysis as follows: 1 ml. of 10% trichloroacetic acid was added to each sample, and the precipitate centrifuged and washed with water. The supernatant fluid and washings were combined, brought to pH 7·5 with 10% NaOH, and diluted to 10 ml. Determinations were then made of total P, acid-labile P (corresponding to glucose-1-phosphate P), and of the fructose content from which the P present as fructose-6-phosphate was calculated. Aldose-6-phosphate P was then calculated as (acid-stable P − fructose-6-phosphate P).

In the control experiment with the boiled extract, 92% of the glucose-1-phosphate was unchanged at the end of the experiment; with the active extract an equilibrium mixture was obtained consisting of 15%

glucose-1-phosphate, 26 % fructose-6-phosphate and 59 % aldose-6-phosphate. Thus phosphoglucomutase and phosphohexoseisomerase were present in the extract.

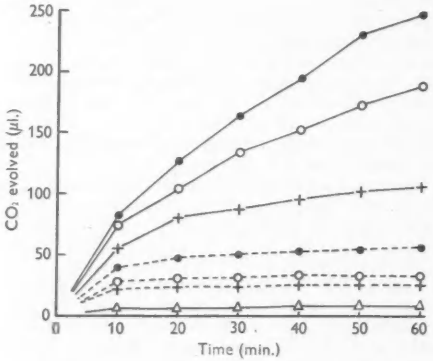

Fig. 1. Activation of the galactose-phosphorylating system in an extract prepared from galactose-adapted Dutch top yeast. The manometers contained—Main chamber: extract, 0·6 ml.; 0·5 M-NaF, 0·2 ml.; 0·12 M-$NaHCO_3$, 0·5 ml.; and, where added, 0·1 M-$MgCl_2$, 0·1 ml.; 0·25 M-cysteine, 0·1 ml. Contents made up to 1·5 ml. with distilled water. Side bulb: 0·1 M-$NaHCO_3$, 0·3 ml.; and, where added, ATP (3·0 mg. $P_{7\ min.}$), 0·4 ml.; galactose (20 mg./ml.), 0·2 ml. Contents made up to 0·9 ml. with distilled water. Gas phase: 95 % N_2 + 5 % CO_2; temp. 25°. ——, mixtures containing galactose; – – – –, mixtures without galactose; ●, ATP, Mg^{++} and cysteine; ○, ATP and Mg^{++}; + ATP only; △, Mg^{++} and cysteine.

The distribution of phosphoric esters in the products of the reaction between adenosinetriphosphate and glucose or galactose

Manometers were set up as described in Fig. 2, and the following quantities were measured during the course of the reaction: (1) increase in acidity, by the CO_2 evolution in bicarbonate buffer, (2) sugar disappearance, (3) change in orthophosphate, and (4) change in acid-labile phosphate.

It can be seen from Fig. 2 that the increase in acidity roughly paralleled the disappearance of either glucose or galactose. With glucose (Fig. 2*A*) there was a decrease of 0·475 mg. in the amount of acid-labile P, corresponding to an esterification of 2·81 mg. glucose, while actually 3·2 mg. glucose disappeared. Thus 87·5 % of the glucose phosphorylated can be accounted for as acid-stable phosphoric esters, presumably an equilibrium mixture of aldose-6-phosphate and fructose-6-phosphate. In the case of galactose, however, there was no significant decrease in the amount of acid-labile P during a period in which 2·1 mg. of galactose disappeared. This suggests that an acid-labile ester such as galactose-1-phosphate was formed.

The disappearance of acid-labile P in the presence of glucose, less that of the control, corresponds to more than one of the acid-labile phosphate groups of ATP. This is supported by the figures for the disappearance of glucose. Since yeast hexokinase is said to be specific to ATP, and to have no reaction with ADP (Colowick & Kalckar, 1943), this result

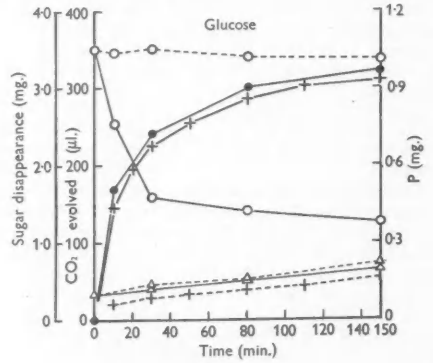

Fig. 2*A*.

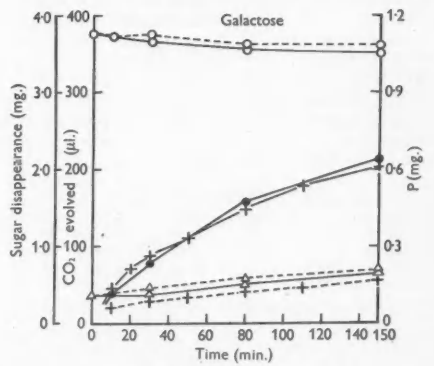

Fig. 2*B*.

Fig. 2. Increase in acidity (CO_2 output), sugar disappearance and phosphate distribution during the breakdown of glucose or galactose by an extract prepared from Dutch top yeast grown on galactose. The manometers contained—Main chamber: extract, 0·6 ml.; 0·5 M-NaF, 0·3 ml.; 0·12 M-$NaHCO_3$, 0·7 ml.; 0·1 M-$MgCl_2$, 0·1 ml.; 0·25 M-cysteine, 0·1 ml.; glucose (20 mg./ml.), 0·3 ml. in *A*; galactose (20 mg./ml.), 0·3 ml. in *B*. Contents made up to 2·1 ml. with distilled water. Side bulb: 0·12 M-$NaHCO_3$, 0·25 ml.; ATP (2·5 mg. $P_{7\ min.}$/ml.), 0·5 ml. Gas phase: 95 % N_2 + 5 % CO_2; temp. 25°. – – – –, control, ——, glucose or galactose; ○—○, phosphate hydrolyzable in 7 min. by N-HCl at 100°; ●—●, free sugar disappearance; +—+, increase in acidity; △—△, orthophosphate.

suggests the presence in the extract of a myokinase-like enzyme catalyzing the reaction

$$2\,ADP \rightarrow ATP + adenylic\ acid.$$

To confirm the production of an acid-labile phosphoric ester from galactose, an experiment was performed in which the distribution of P among the various phosphoric esters was determined by separation into soluble and insoluble barium salts, and estimation of inorganic P, acid-labile P, and total P in each fraction.

The results are shown in Table 2 Exp. A. When glucose is the substrate, the ATP-P which disappears (decrease in acid-labile P of insoluble barium salts), is largely accounted for in the soluble barium salts as an acid-stable ester (probably a mixture of aldose-6-phosphate and fructose-6-phosphate), and only a small amount of an acid-labile ester is found in this fraction (probably glucose-1-phosphate). When galactose is the substrate, the situation is reversed, and the major part of the ATP-P disappearing is recovered in the soluble fraction as an acid-labile ester. The proportions of acid-labile to acid-stable P in the soluble barium salts are 0·14:0·86 and 0·87:0·13 for glucose and galactose respectively.

The experiment described in Table 2, Exp. A, was repeated using an extract prepared from non-adapted Dutch top yeast. In this case, 96 % of the galactose remained unchanged after 150 min. and the CO_2 evolution was 165 μl. compared with 161 μl. in the control. In the same time, 74 % of the glucose disappeared and the corresponding CO_2 evolution was 871 μl. Thus it appears that Dutch top yeast grown on glucose will not phosphorylate galactose. Berger, Slein, Colowick & Cori (1945–6) have shown that purified yeast hexokinase will not attack galactose.

Isolation of α-galactose-1-phosphate

The reaction mixture contained: 40 ml. ATP solution (approx. 6 mg. $P_{7\ min.}$/ml.); 1 g. galactose; 2 ml. M-cysteine; 5 ml. 0·1 M-$MgCl_2$; 20 ml. 0·5 M-NaF; 40 ml. 0·12 M-$NaHCO_3$; 40 ml. extract, and water to a total volume of 150 ml. Portions (50 ml.) were placed in each of three Krebs (1933) vessels which were filled with a N_2/CO_2 mixture containing 5 % CO_2 and shaken in a bath at 25°. The reaction was allowed to proceed until no more galactose disappeared. Trichloroacetic acid (20 ml. of 40 %) was then added to the combined contents of the vessels, and the precipitate centrifuged and washed with 5 % trichloroacetic acid. The supernatant and washings were brought to pH 8·5 with 10 % NaOH, and barium acetate (50 ml. of 25 %) added. The precipitate was centrifuged and the supernatant poured off and kept. The precipitate was then dissolved in 25 ml. 0·1 N-HCl, the pH brought to 8·5 with 10 % NaOH and the precipitate centrifuged and discarded. The double extraction was necessary since the first precipitate contains some 'barium-soluble' material in the form of double salts (Cori & Cori, 1932). Two vol. ethanol were added to the combined supernatants, and the mixture left at 0° for 2 hr. The precipitate was then centrifuged, washed in turn with 75 % ethanol, 96 % ethanol and ether and dried *in vacuo* over $CaCl_2$. From 0·57 g. galactose metabolized, 3·18 g. of crude soluble Ba salt were obtained, containing 124·1 mg. P of which 73·4 mg. was acid-labile.

The crude Ba salts contained an impurity of carbohydrate nature, which was removed by taking advantage of the solubility of the K salts of hexosemonophosphates at pH 6·0 in 60 % ethanol; under these conditions, polysaccharides are

Table 2. *Distribution of phosphoric esters formed by the action of an extract prepared from galactose-adapted Dutch top yeast on a mixture of galactose or glucose and adenosinetriphosphate*

(Exp. A, normal extract; initial acid-labile P = 3·62 mg. Exp. B, extract prepared at 0°; initial acid-labile P = 3·21 mg. The manometers contained—Main chamber: 0·5 M-NaF, 0·3 ml.; 0·1 M-$MgCl_2$, 0·3 ml.; 0·25 M-cysteine, 0·3 ml.; 0·12 M-$NaHCO_3$, 1·4 ml.; ATP (3–4 mg. $P_{7\ min.}$/ml.), 1·0 ml.; and where added, glucose or galactose (20 mg./ml.), 0·8 ml. Contents made up to 4·1 ml. with distilled water. Side bulb: extract, 0·5 ml.; 0·5 M-NaF, 0·1 ml.; 0·12 M-$NaHCO_3$, 0·3 ml. Gas phase: 95 % N_2 + 5 % CO_2; temp. 25°. CO_2 output followed until complete (150 min. in A, 280 min. in B). Contents of manometers then separated into soluble and insoluble barium salts and analyzed.)

	Soluble Ba salts					Insoluble Ba salts				Total P	
	Inorganic P (mg.)	Acid-labile P (mg.)	Acid-stable P (mg.)	Increase in acid-labile P* (mg.)	Increase in acid-stable P* (mg.)	Inorganic P (mg.)	Acid-labile P (mg.)	Acid-stable P (mg.)	Decrease in acid-labile P* (mg.)	Initial (mg.)	Recovered (mg.)
					Exp. A						
Control	0·00	0·24	0·48	—	—	0·74	2·86	1·92	—	6·81	6·24
Glucose	0·04	0·42	1·78	0·18	1·30	0·74	1·38	1·68	1·48	6·81	6·04
Galactose	0·02	1·28	0·64	1·04	0·16	0·76	1·80	1·96	1·06	6·81	6·26
					Exp. B						
Control	0·00	0·12	0·19	—	—	0·31	2·50	1·56	—	4·82	4·37
Glucose	0·00	0·18	2·29	0·06	2·10	0·28	0·67	1·26	1·83	4·82	4·34
Galactose	0·00	1·13	1·00	1·01	0·81	0·36	0·96	1·16	1·54	4·82	4·25

* Compared with control.

precipitated. The salt was taken up in 200 ml. water, the insoluble material being discarded, the Ba^{++} removed from the supernatant by addition of a slight excess of saturated K_2SO_4, and the pH adjusted to 6·0 with 10% KOH; 3 vol. ethanol were then added. The mixture was left at 0° overnight, and the precipitate discarded. The supernatant was concentrated *in vacuo* to 40 ml., and the precipitate which formed again discarded. The supernatant was brought to pH 8·4 with 10% NaOH, and 5 ml. 25% barium acetate and 2 vol. ethanol were added. After leaving at 0° overnight, the precipitate was washed and dried in the usual manner; wt. 1·281 g.; P, 5·1%, of which 88·6% was acid-labile. The recovery of acid-labile P from the crude Ba salt was 79·2%. A sample of the material, after treatment with $Ba(OH)_2$ and $ZnSO_4$, was free from Cu-reducing substance. Another sample was hydrolyzed for 7 min. in N-HCl, and the sugars so produced were subjected to a differential fermentation. Ba salt (50 mg., containing 2·27 mg. acid-labile P) yielded 12·9 mg. of reducing sugar (equivalent to 2·25 mg. P), of which only 8% was fermented by glucose-grown yeast and a further 92% by galactose-grown yeast. It is assumed, therefore, that 8% of the acid-labile P was present as a glucose ester, and 92% as a galactose ester. The salt contained negligible amounts of pentose and fructose, the acid-stable P probably being present largely as aldose-6-phosphate.

Any reducing phosphates were removed by alkaline hydrolysis as follows. The salt (1·136 g.) was dissolved in 100 ml. water and Ba^{++} removed with 5 ml. N-H_2SO_4. The precipitate was centrifuged and washed with 5 ml. water. To the supernatant and washings were added 27 ml. N-NaOH and hydrolysis was allowed to proceed at 100° for 30 min. After cooling, the solution was brought to pH 8·4 with 2N-HCl, 25 ml. 25% barium acetate added, and the precipitate collected in the usual manner. Further purification was effected by repeated solution in water and precipitation with ethanol. The final product was identified as barium α-galactose-1-phosphate as follows.

Identification of the product as barium α-galactose-1-phosphate. The salt was dried over P_2O_5 *in vacuo* to a constant weight of 474 mg.; P, 7·49%; acid-labile P, 7·47%. (Calc. for $C_6H_{11}O_9PBa$: P, 7·85%.) The recovery of acid-labile P from the crude barium salt was 48·1%. The product contained less than 0·1% N, no detectable reducing power as measured by the method of Somogyi (1945), and negligible amounts of pentose and ketose. Further evidence on its identity was obtained as follows.

(1) *Sugar liberated on hydrolysis.* Hydrolysis in N-HCl at 100° for 7 min. resulted in the simultaneous liberation of reducing sugar and inorganic P in equimolecular amounts. On hydrolysis and differential fermentation of the sugars liberated, 25 mg. Ba salt yielded 0·6 mg. 'glucose' (2·4%), 10·2 mg. 'galactose' (40·8%), and 1·76 mg. inorganic P, giving a molecular ratio galactose : acid-labile P of 0·99 : 1·00. (Barium galactose-1-phosphate requires galactose, 45·5%.)

(2) *Specific rotation.* The Ba salt had $[\alpha]_{5461}^{20°} = +110°$. This agrees well with the value found by Kosterlitz (1943*a*) of 109·5° for the Ba salt of synthetic α-galactose-1-phosphate, and with that of 113° for the Ba salt of the natural ester calculated by the same author from the rotation of an impure preparation. Reithel (1945) found the specific rotation of the barium salt of β-galactose-1-phosphate to be $[\alpha]_D^{30°} = +34°$.

(3) *Hydrolysis constant.* Hydrolysis was carried out in 0·25N-HCl at 25° using the K salt of the ester, the galactose liberated being measured by the Somogyi (1945) method and the inorganic P by the Fiske & Subbarow (1925) method.

The hydrolysis constant in 0·25N-HCl at 25° was $0·86 \times 10^{-3}$ (Table 3). The corresponding value for the K salt of synthetic α-galactose-1-phosphate was found by Kosterlitz (1939) to be $0·89 \times 10^{-3}$, and the same author (Kosterlitz, 1943*a*) calculated the hydrolysis constant for the K salt of the natural ester from the hydrolysis rate of an impure preparation to be $0·91 \times 10^{-3}$.

Table 3. *Acid hydrolysis of the acid-labile galactose ester*

(Hydrolysis at 25° in 0·25N-HCl. Initial concentration of acid-labile P = 2·93 mM. Initial concentration of galactose = 2·88 mM. The hydrolysis constant k was calculated from the formula $k = \frac{1}{t_2 - t_1} \log_{10} \left(\frac{a - x_1}{a - x_2} \right)$.

Time (min.)	Galactose liberated (% of total galactose)	$k \times 10^3$	Inorganic P liberated (% of total acid-labile P)	$k \times 10^3$
100	18·2	0·87	18·3	0·88
200	32·7	0·85	32·9	0·86

On the basis of this evidence it is concluded that the easily hydrolyzable ester formed from galactose and ATP is α-galactose-1-phosphate.

Possible formation of galactose-6-phosphate as an intermediate

To test the possibility that the primary phosphorylated product in galactose fermentation is galactose-6-phosphate, which is immediately transformed to galactose-1-phosphate, the effect of adding synthetic galactose-6-phosphate to the enzyme system phosphorylating galactose was studied. The results are shown in Table 4. In the presence of galactose-6-phosphate, the distribution of phosphoric esters did not differ significantly from that of the control, and there was no diminution in the amount of acid-stable P during the course of the reaction. These facts, together with the absence of increased acid formation (CO_2 evolution) over the control, indicate that galactose-6-phosphate is not attacked in a system which is able to form an easily hydrolyzable phosphoric ester from galactose. Galactose-6-phosphate is, therefore, not an intermediate in the formation of galactose-1-phosphate in this system.

The action of the enzyme system on D-tagatose, the ketose corresponding to D-galactose, was also investigated. Tagatose was not fermented by intact cells of galactose-adapted yeast, but this does not necessarily exclude it from playing a part in galactose fermentation analogous to that of fructose in glucose fermentation. It can be seen, however, from Table 4, that tagatose was not phosphorylated by an extract of galactose-adapted cells. The figures for the P distribution in the presence of tagatose were almost identical with those in the control.

Table 4. *Distribution of phosphoric esters formed by the action of an extract prepared from galactose-adapted Dutch top yeast on a mixture of galactose, galactose-6-phosphate or tagatose, and adenosinetriphosphate*

(The manometers contained—Main chamber: 0·5 M-NaF, 0·3 ml.; 0·1 M-$MgCl_2$, 0·3 ml.; 0·25 M-cysteine, 0·3 ml.; 0·12 M-$NaHCO_3$, 1·4 ml.; ATP (4·0 mg. $P_{7\ min.}$/ml.), 1·0 ml.; and, where added, galactose or tagatose (20 mg./ml.) or galactose-6-phosphate (1·88 mg. P/ml.), 0·8 ml. Side bulb: extract, 0·5 ml.; 0·5 M-NaF, 0·1 ml.; 0·12 M-$NaHCO_3$, 0·2 ml. Gas phase: 95 % N_2 + 5 % CO_2; temp. 25°. CO_2 output was followed until complete (210 min.), and the phosphate esters formed were then separated into soluble and insoluble barium salts and analyzed.)

	Initial P		Soluble Ba salts				Insoluble Ba salts			
	Control: galactose or tagatose (mg. P)	Galactose-6-phosphate (mg. P)	Control (mg. P)	Galactose (mg. P)	Galactose-6-phosphate (mg. P)	Tagatose (mg. P)	Control (mg. P)	Galactose (mg. P)	Galactose-6-phosphate (mg. P)	Tagatose (mg. P)
Ortho-P	0·00	0·00	0·00	0·00	0·00	0·00	0·23	0·24	0·23	0·21
Acid-labile P	3·92	3·92	0·14	1·52	0·16	0·14	3·19	1·60	3·23	3·07
Change in acid-labile P*	—	—	—	+1·38	+0·02	0·00	—	−1·59	+0·04	−0·12
Acid-stable P	2·15	3·65	0·25	0·29	1·70	0·25	1·45	1·37	1·55	1·50
Change in acid-stable P*	—	+1·50	—	+0·04	+1·45	0·00	—	−0·08	+0·10	+0·05
CO_2 in 210 min. (μl.)	—	—	210	726	214	221	—	—	—	—

* Compared with control.

The production of acid-stable phosphoric esters from galactose

It was found that if precautions were taken to keep the temperature down to 0° during the breakdown of the yeast cells and the purification of the enzyme, there was occasionally obtained from galactose an acid-stable ester which appears in the soluble barium salts. Typical results for the distribution of P with such an extract are given in Table 2, Exp. B. While the phosphorylation of glucose results in the formation of an acid-stable ester (2·10 mg. P), that of galactose gave a mixture of an acid-labile ester (1·01 mg. P) and an acid-stable ester (0·81 mg. P). It seems that if these precautions about cooling are taken, a second adaptive enzyme, which is destroyed during the more drastic procedure, is present and forms an acid-stable ester from galactose-1-phosphate.

Using an extract prepared with particular attention to adequate cooling, an attempt was made to isolate the acid-stable ester formed from galactose. The preparation was set up in the same way as for the isolation of α-galactose-1-phosphate described above, but using half the quantities. After 4 hr., 210 mg. of galactose had disappeared.

The soluble Ba salts were isolated as described previously: wt. 907 mg.; P, 5·9 %, of which 24·9 % was acid-labile and 75·1 % acid-stable. Differential fermentation of the sugar produced by hydrolysis in N-HCl at 100° for 7 min. showed it to consist of 86 % 'galactose' and 14 % 'glucose', and, therefore, the acid-labile ester was probably chiefly galactose-1-phosphate. The Ba salt (500 mg. ≡ 22·2 mg. acid-stable P) was converted into the Na salt (120 mg.), and 15 ml. 5 N-HCl added. The acidified solution was boiled for 7 min. to hydrolyze the acid-labile phosphoric esters present, cooled to room temp., the pH brought back to 8·4 with 10 % NaOH, and 3 ml. 25 % barium acetate added. The precipitate was centrifuged, washed with 10 ml. water, and 2 vol. 96 % ethanol were added to the combined supernatants. The precipitate was centrifuged, washed with ethanol and ether, and dried over $CaCl_2$ *in vacuo*. The salt was redissolved in 25 ml. water, the insoluble material being spun off, reprecipitated with 2 vol. ethanol, washed with ethanol and ether, and dried over $CaCl_2$ *in vacuo*; wt. 212 mg.; P, 6·18 %; acid-labile P, nil. (Recovery of acid-stable P = 59 %.)

The pentose and ketose in the precipitate were negligible, and the total N was less than 0·1 %. The reducing value of the salt, estimated by a Somogyi copper reagent standardized against glucose-6-phosphate, was equivalent to a content of 59·5 % barium glucose-6-phosphate (corresponding to 78·2 % of the total acid-stable P). No insoluble methylphenylhydrazone could be obtained from the barium-free esters, showing that galactose-6-phosphate was not a constituent (Grant, 1935).

The ester was hydrolyzed by a non-specific phosphatase prepared from a dialyzed extract of homogenized rat intestine. A solution of the Na salt (10 ml. containing 3·24 mg. P) was incubated for 2 hr. at 37° with 5 ml. extract, 25 ml. acetate buffer (pH 5·5), and 1 ml. 0·1 M-$MgCl_2$; the inorganic P liberated corresponded to 89·5 % of the total P and the P:sugar ratio was 1·00:0·705. For comparison, a similar mixture was set up using sodium glucose-6-phosphate (prepared from 50 mg. anhydrous barium glucose-6-phosphate) in place of the unknown Na salt; the inorganic phosphate liberated was 84 % of the total, and the P:sugar ratio was 0·91:1·00.

These figures, together with those given in the previous paragraph, suggest that the unknown salt was a mixture of acid-stable esters, one of which is non-reducing and produces a non-reducing substance on hydrolysis, and the other is reducing and contains a reducing sugar. It has not yet been possible to

investigate these esters more completely, but it seems reasonable to suppose that the reducing ester is an aldose-6-phosphate, probably glucose-6-phosphate. The non-reducing ester may be trehalose-monophosphate (Robison & Morgan, 1930; Grant, 1935).

The formation of galactose-1-phosphate during adaptation to galactose fermentation

Reiner (1947) investigated the change in acid-labile P during the period immediately preceding the onset of adaptation to galactose fermentation by *Saccharomyces cerevisiae*, and concluded that fermentation was preceded by the accumulation of ATP. He did not separate the phosphoric esters into soluble and insoluble barium salts, however, and in view of the findings presented in this paper, his results might equally well have been explained by the accumulation of galactose-1-phosphate in the prefermentative period, thus stimulating the formation of a second adaptive enzyme attacking galactose-1-phosphate with the production of an acid-stable phosphoric ester. This point was investigated by the author using a separation into soluble and insoluble barium salts to differentiate between ATP and galactose-1-phosphate.

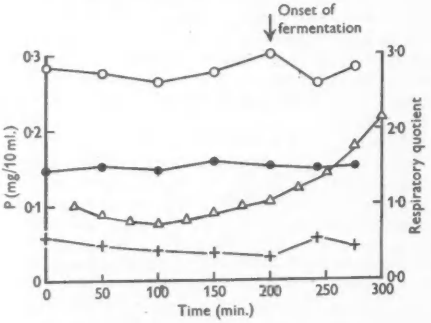

Fig. 3. Distribution of phosphoric esters during the adaptation of Dutch top yeast to the fermentation of galactose. ○—○, phosphorus hydrolyzable in 7 min. in the insoluble barium salt fraction; ●—●, orthophosphate; +—+, phosphorus hydrolyzable in 100 min.—alkali-labile phosphorus in the soluble barium salt fraction; △—△, respiratory quotient.

The separation was carried out according to the method of Umbreit *et al.* (1945), and inorganic phosphate was estimated by the method of Berenblum & Chain (1938). The remainder of the experiment was carried out as described by Reiner (1947), but using Dutch top yeast and incubating at 25°. The results are shown in Fig. 3. It can be seen that, in the period immediately preceding adaptation (fermentation), there was no accumulation of P in the fraction ($P_{100} - P_{\text{alkali labile}}$) which would have contained galactose-1-phosphate.

DISCUSSION

From the work described in this paper, it seems reasonably certain that the first step in galactose fermentation by Dutch top yeast is the transference of the terminal phosphate group from ATP to galactose to give galactose-1-phosphate. The enzyme catalyzing this reaction is activated by Mg^{++} and by cysteine, and clearly belongs to the class of phosphokinases. It is proposed to call it galactokinase.

By analogy with hexokinase it might be expected that galactose-6-phosphate would be the primary product, but this seems very unlikely for the following reasons: (1) no yeast extract yet investigated can ferment galactose-6-phosphate (Hvistendahl, 1932; Grant, 1935); (2) the enzyme system which forms galactose-1-phosphate from galactose is unable to attack galactose-6-phosphate (Table 4); (3) there is no initial formation of an acid-stable ester during the phosphorylation of galactose by the extract (Fig. 2); (4) the extract is unable to phosphorylate tagatose, whereas yeast hexokinase will phosphorylate fructose and mannose in addition to glucose.

It has been shown by Spiegelman, Reiner & Morgan (1947) that the acquisition of the ability to ferment galactose by *Saccharomyces cerevisiae* involves the formation of one or more apoenzymes. The work described in this present paper shows that one of these enzymes is galactokinase.

Since galactose is fermented with the formation of fructose-1:6-diphosphate (Grant, 1935), the question arises as to how the galactose-1-phosphate is transformed into hexosediphosphate. The answer to this question is still obscure, but some indication is given by the work of Kosterlitz (1943*b*), and by the experiments described in this paper in which a proportion of the phosphate esterified was present as an acid-stable phosphoric ester with a soluble barium salt. If, as seems probable, this proves to be glucose-6-phosphate, then it seems reasonable to suppose that the transformation of galactose-1-phosphate to fructose-1:6-phosphate takes place via glucose-6-phosphate or an ester in equilibrium with glucose-6-phosphate in the system described.

SUMMARY

1. Dutch top yeast has been shown to produce adaptively an enzyme, named galactokinase, which catalyzes the phosphorylation of galactose by adenosinetriphosphate forming an ester which has been identified as α-galactose-1-phosphate. The enzyme requires Mg^{++} and cysteine for full activity.

2. Galactose-6-phosphate is not an intermediate in this reaction.

3. The formation of an acid-stable ester from galactose has been observed occasionally with extracts of galactose-adapted yeast.

4. There is no evidence for the accumulation of galactose-1-phosphate during the pre-adaptive period.

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Studies on the Lens

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1. POST-MORTEM CHANGES IN THE MINERAL CONTENT

Our knowledge of the composition and metabolism of the lens has been gained almost exclusively from work on the excised organ, and it is therefore of first importance that post-mortem changes should be fully understood. It might be thought that the use of normal aqueous humour as a bathing medium for the excised lens would make such a study supererogatory, but the results described below show that, even when the lens is left undisturbed in the dead eye, important changes in its mineral content (characteristic of those taking place in a dying tissue) occur within a few hours.

EXPERIMENTAL

To minimize variance due to age, adult rabbits were used in all the experiments described with this species. Except where otherwise stated, the sheep eyes were received from the slaughterhouse within approximately 2 hr. of death. After removing the aqueous humour and cutting away the posterior half of the eye the lens was removed by placing the anterior half of the eye on a flat dish, cornea downwards, reflecting back the curved scleral wall and cutting the suspensory ligament. Before being weighed, the lens was blotted on filter paper. For analysis, the proteins were precipitated by the Somogyi (1930) method and the filtrate made up to a definite volume. Chloride was estimated by the $AgIO_3$ method of Sendroy (1937). When radioactive isotopes were used, activity was measured in the filtrate on a standard Geiger counter apparatus with the counter described by Maurice (1948). Radioactive sodium (^{24}Na) in the form of NaCl, and bromine (^{82}Br) in the form of NH_4Br were injected intravenously in isotonic solutions, without anaesthesia. After the experimental period the animal was killed by a blow on the neck, and the eyes, together with blood samples, were taken immediately.

For the *in vitro* study of the 'diffusion out' of chloride from the lens, the latter was placed in isotonic Na_2SO_4 solution, while for the 'diffusion in' of sucrose, aqueous humour was diluted with 0·3 vol. of an isotonic sucrose solution. Sucrose was determined by hydrolyzing the Somogyi filtrate of the macerated lens with dilute H_2SO_4, and estimating the reducing value by the Hagedorn-Jensen method.

RESULTS

As a criterion for the normal condition of the lens the chloride content was first chosen. Preliminary experiments revealed that the chloride contents of the lenses of a pair of eyes removed immediately after death did not differ significantly; thus, the mean of the chloride contents of ten left sheep eyes was 43·8 ± 0·2 whilst that for the right eyes was 43·7 ± 0·2. In the first study, therefore, the two eyes were excised from the freshly killed animal; the lens of one was submitted to analysis immediately, whilst that of the other was analyzed after it had remained in the excised eye for a period of some hours. In Table 1 the chloride contents of the pairs of eyes are

Table 1. *Post-mortem changes in the chloride content of lenses allowed to remain in the excised eye*

Species	Time test lens left in eye (hr.)	Temp. (°)	Chloride content (mg./100 g.) Control lens	Test lens
Rabbit	2	0	31·9	31·0
	2	0	32·1	33·7
	4	0	30·7	37·3
	4	0	29·9	35·1
	5	0	33·3	37·1
	4·5	0	31·0	33·9
	6·5	0	25·6	31·0
	7·0	0	36·4	39·3
	5·25	20	33·6	46·3
		Mean	**34·0 ± 0·79**	**39·5 ± 1·14**
Sheep	6·0	20	39·4	45·9
	6·0	20	33·1	43·9
	3·0	20	35·6	37·6
	3·0	20	38·2	43·0
		Mean	**36·6 ± 0·64**	**42·6 ± 1·0**

shown. The results indicate that the lens left in the eye takes up chloride; thus, for rabbits, the mean chloride content of the control eyes was 34·0 mg./100 g. whilst that of the eyes left for more than 2 hr. was 39·5 mg./100 g.; it will be noted that the lenses left for only 2 hr. at 0° showed no increase in chloride content. There is thus a post-mortem increase in the chloride content of the lens even when it is allowed to remain in the eye. It is generally assumed that the chloride content of a normal tissue is a measure of the extracellular space, the cells being virtually free from this ion; the increase in chloride content observed may, therefore, be interpreted as the result either of an increase in extracellular space or of degenerative changes in the lens fibres which permit them to accumulate chloride. Changes in the chloride content should be paralleled by changes in the sodium content on the basis of either of these explanations. Consequently, the uptake of radioactive sodium by the lens *in vivo* should be less than the uptake of another, excised, lens from an artificial medium. Similarly, by use of the radioactive bromide ion, differing uptakes should be obtained under the two conditions. In Table 2 the results of diffusion experiments are shown; they indicate that when diffusion takes place from an artificial medium

Table 2. *Ratio of the concentrations of isotopes in lens and in aqueous humour* in vitro *and* in vivo *studies*

(For *in vitro* studies the lens was suspended in aqueous humour containing the isotope. For *in vivo* studies the isotope was injected intravenously into the animal without anaesthesia. The results are calculated from counts/100 g. of material and are expressed as mean ± S.E. The number of experiments is given in brackets.)

Type of exp.	Diffusion time (hr.)	Mean ratio: isotope in lens/isotope in aqueous humour
In vitro; ^{24}Na	24	0·29 ± 0·01 (6)
	48	0·36 ± 0·07 (6)
In vitro; ^{82}Br	24	0·29 ± 0·03 (6)
	40	0·29 ± 0·03 (6)
In vivo; ^{24}Na	2	0·03 ± 0·02 (6)
	24	0·06 ± 0·01 (4)
	41–46	0·05 ± 0·02 (6)
In vivo; ^{82}Br	24–48	0·07 ± 0·02 (4)

over a period of 24–48 hr. at 0° the ratio Na in lens/Na in aqueous humour is 0·29–0·36, whereas *in vivo* experiments gave very much smaller ratios in the region of 0·07 when the diffusion time was either 24 or 48 hr. Similarly, results with the radioactive bromide ion gave large *in vitro* ratios in the region of 0·29 and *in vivo* values of approximately 0·065.

DISCUSSION

The primary importance of the experimental findings described here must be in emphasizing the limitation of *in vitro* experiments on the lens under the given conditions. In some of the experiments described the lens was merely left in the excised eye, i.e. it was surrounded by intraocular fluid during the whole time, and yet quite unmistakable changes in its chloride content took place in a few hours. In the living eye there is strong reason to believe that the aqueous humour flows continuously, being drained away at the angle of the anterior chamber to be replaced by new fluid presumably from the ciliary body; the cessation of this renewal is apparently sufficient, even with a tissue of such low metabolic activity as the lens, to cause changes in the salt content of the lens that are generally interpreted as degenerative, i.e. a breakdown of the normal permeability of the fibre cells to sodium. It is interesting that in a recent publication Palm (1948), working on the penetration of radioactive phosphorus into the lens, came to the conclusion that experiments not completed within 2 hr. of the death of the animal were altogether unreproducible.

Although much experimental work has been done on the lens and isolated capsule there seem to be few detailed investigations of the speed of post-mortem changes. On the other hand, the literature is well stocked with references to the changes in composition that occur with development of cataract, changes indicating breakdown of the normal ion permeability of the lens fibres to the sodium chloride in their environment. It is not yet possible to correlate this increase in permeability with specific metabolic changes, but consideration of the following is of interest. Experimental work of the authors (unpublished) supports the results in the literature (Bellows, 1944) indicating that there is no significant decrease of carbohydrate in the lens or aqueous humour during the first few hours following death. The deduction that the presence of glucose does not delay the degenerative changes is supported by the inability of a Krebs-Ringer phosphate medium containing glucose, or an aqueous medium to which isotonic glucose solution has been added, to retard the speed of these changes (unpublished observations).

Much experimental evidence has led to general agreement that the energy required by the normal lens is obtained primarily from carbohydrate metabolism (Bellows, 1944; Fischer, 1930), and further that, unlike other active tissues, the lens has no carbohydrate reserve in the form of glycogen (Weekers & Sullman, 1938).

If we accept these conclusions it is apparent that, following death, a factor is introduced which leads to interference in the utilization of sugar. The importance of an active endergonic metabolism in the normal tissue is further supported by the slowing of the rate of post-mortem changes following a decrease in the temperature of the lens.

2. ESTIMATION OF EXTRACELLULAR SPACE OF LENS

The extracellular volume of muscle has been calculated from its sodium and chloride contents on the well-supported assumption that these ions exist mainly or totally outside the cell; additional methods have involved the study of the uptake of a large water-soluble molecule like that of sucrose which is presumed not to penetrate the cell membrane.

The chloride contents of the rabbit and sheep lens were found by analysis to be $34{\cdot}7 \pm 0{\cdot}6$ mg./100 g. (10) and $43{\cdot}7 \pm 0{\cdot}6$ mg./100 g. (20) (numbers in brackets refer to numbers of experiments). The aqueous humour concentrations of the same eyes, expressed as mg./100 ml., were 397 for the rabbit (6) and 460 for the sheep (14). Consequently the chloride space, i.e. the fraction of the weight occupied by interstitial fluid, assuming that all the chloride is present in the latter, is 9·5 % for the sheep and 8·7 % for the rabbit. In view of the unique growth characteristics of the lens, however, it is doubtful whether this fraction does indeed represent the true extracellular space; thus, the lens continues cell proliferation throughout life, the newly formed surface cells displacing the older ones inwards towards the centre, a process that results in the formation of a hard nucleus of old, densely packed fibres and a softer cortex of more recently formed elements. As a result of this development it is quite possible that the fibres of the densely packed nucleus, representing very old cells, contain appreciable amounts of sodium and chloride. If this is true the estimate of the extracellular space from the chloride content must give too high a value. Moreover, the cells containing sodium and chloride will be predominantly in the dense highly viscous nucleus, and will be therefore unable to attain diffusion equilibrium with their environment within an experimentally measurable period. In the present work the fraction of the lens that comes into apparent diffusion equilibrium, in respect to chloride, ^{24}Na, ^{82}Br and sucrose with its environment within 24–48 hr., has been determined. We may call this the 'readily available space'. In so far as the chloride ion was concerned, the space was estimated on the basis of the amount that diffused out of the lens into an isotonic sulphate solution within 24–48 hr. For ^{24}Na the results already described on the diffusion into the lens *in vivo* were used for the computation. Sucrose was allowed to diffuse into the excised lens from an aqueous humour medium; in view of the post-mortem changes already described we may expect this substance to provide altogether too high a value for the 'readily available space'. This was indeed found to be the case; washing the lens, after the diffusion in sucrose, for 0·5 hr. in a sulphate medium reduced the 'sucrose space' to a value comparable with the 'readily available chloride space'.

RESULTS

The results of this investigation are summarized in Table 3. It will be seen that the 'readily available chloride space', determined by the fraction of chloride that diffuses out of the lens in 24–48 hr., is 5·5 % as opposed to the value of 8·5–9·6 % for the total chloride space. On the basis of *in vivo* diffusion of radioactive isotopes into the lens the 'readily available space' is 6·1–6·7 %. Sucrose diffuses into an available space of 13·5 %, indicating the occurrence of post-mortem changes. If the lenses, after the diffusion of sucrose into them, are placed in a sucrose-free medium, there is an initial rapid diffusion of sucrose out; this rapid loss is succeeded by a much slower loss during the next 24 hr. If the sucrose space is computed on the basis of the sucrose content after this rapid loss it averages 5·5 %.

Table 3. *Values of the extracellular volume of lens*

Method	Total space (%)	Readily available space (%)
Chloride analysis and diffusion out into Na_2SO_4	9·6 (sheep) 8·5 (rabbits)	5·5 (sheep) —
Diffusion of ^{24}Na *in vivo*	—	6·1 (rabbits)
Sucrose (diffusion into lens)	13·5 (sheep)	—
Sucrose (diffusion into lens and subsequent washing in isotonic Na_2SO_4)	—	5·46 (sheep) —
Diffusion of ^{82}Br *in vivo*	—	6·7 (rabbits)

DISCUSSION

For kinetic studies on the penetration of substances from aqueous humour into the lens fibres a knowledge of the magnitude of the effective extracellular space—the 'readily available space' discussed here—is a first essential. The results described in this paper indicate that this space is of the order of 6–7 % where *in vivo* studies are concerned. Owing to its greater density, the nucleus of the adult lens contains a higher concentration of potassium (expressed in mg./unit wt. of lens) than the cortex; the ratio [K]/[Na] in the nucleus is, however, lower than that in the cortex (Bellows, 1944). If it is assumed that sodium is predominantly an extracellular ion, it might be deduced that the nucleus has a greater extracellular matrix. This conclusion appears doubtful on histological grounds since the hard, viscous, nucleus is composed of densely packed fibres. A more probable explanation for the lower ratio is that the nuclear fibres are partly permeable to sodium, thereby permitting sodium and chloride to displace potassium, an explanation with which the present findings are in agreement. It is apparent that the nucleus must have a very low metabolism indeed and this fact, coupled with the disappearance of the cell nuclei from these central cells, would support the view of an increased permeability to sodium.

SUMMARY

1. The limitation of the *in vitro* experiment on the excised lens is deduced from studies of the rate at which post-mortem changes occur.

2. Three methods for the determination of the 'readily available space' are described.

3. Analysis of the results shows that this space is 5·0–5·5 % of the volume of an adult sheep's lens and approximately 6 % of the volume of an adult rabbit's lens.

4. From an analysis of the K/Na ratio in the nucleus and the cortex, together with anatomical and histological evidence, it is suggested that the chloride that fails to diffuse out of the lens within 24–48 hr. is contained in the nucleus.

We are grateful to the Medical Research Council for a grant to one of us (M.L.) and for defraying the whole cost of this work.

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Breakdown of Cozymase by a System from Nervous Tissue

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Cozymase has been observed to be unstable in many biological systems (Harden & Young, 1906*a*, *b*; Schlenk, 1945; McIlwain, 1947; McIlwain & Hughes, 1948). In brain tissue Mann & Quastel (1941) and Handler & Klein (1942) showed the loss of cozymase to be due to a system which liberated nicotinamide from the coenzyme, and which was inhibited by added nicotinamide. Since then many investigators have taken precautions to prevent the breakdown when using systems dependent on cozymase, but the breakdown has not been studied sufficiently for an assessment to be made of its significance in the normal activities of nervous tissue. Because of the important part played by cozymase itself, we considered it especially necessary to determine the speed of the breakdown, and its variation with cozymase and nicotinamide concentrations and with other factors likely to affect it in the living organism. The breakdown has been found to be very rapid, and to involve the formation of acid (see equation I, p. 474).

EXPERIMENTAL

Materials

Cozymase specimens. Pure cozymase has not been obtained, but the reaction has been carried out with three different specimens of the substance, for which analytical data are given in Table 1. Specimen D was used in the majority of experiments. Specimen A, Table 1, is specimen A of Table 1 of McIlwain & Hughes (1948), and further data concerning it are given in that paper. The determinations of catalytic activity quoted in Table 1 were made in the apozymase system under conditions previously described (McIlwain & Hughes, 1948). The nicotinamide contents quoted for specimens D and E were obtained by liberating the amide by excess of the brain enzyme, and determining the nicotinamide colorimetrically (see below).

Table 1. *Analytical data concerning cozymase specimens*

	Determination and standard value	Cozymase specimen A	D	E
I	Acid formation in reaction with $Na_2S_2O_4$ (Warburg & Christian, 1936) (μmol./mg.)	1·75	2·76	2·58
	Purity by comparison with theoretical value of 4·53 μmol./mg. ($C_{21}H_{27}O_{14}N_7P_2$; mol. wt. 663) (%)	39	61	57
II	Nicotinamide content (%) (theoretical value 18·6)	8·0	11·5	9·9
	Purity (%)	43	62	53
III	Catalytic activity in apozymase system (μmol. CO_2/μg./hr.)	1·47	2·40	—
	Purity (by comparison with value of 4·0 derived from Axelrod & Elvehjem, 1939 and McIlwain & Hughes, 1948) (%)	37	60	—
	Origin of specimens	This laboratory	Schwartz Laboratories, Inc.	

Tissue preparations. Guinea pigs, rabbits and rats were killed by a blow on the neck, sometimes bled, the skull opened and the brain put on ice, or ground, within 3–4 min. of death. Sheep brain and cord were obtained from a slaughterhouse as soon as possible after killing and brought to the laboratory in ice water. Tissue was ground by hand with an equal weight of acid-washed sand in a cold mortar or homogenized in the glass apparatus of Potter (see Umbreit, Burris & Stauffer, 1945). Crushed tissue was prepared by pressing a fragment approx. 3 × 3 × 2 mm. to approx. 0·3 mm. thickness between slides; it was then handled as a slice. A teased preparation of white matter was made by drawing a fine needle through the material in the direction of the fibres.

Ground preparations with which several experiments were to be performed were prepared as follows (cf. Handler & Klein, 1942). Tissue (25 g.) and sand (25 g.) were rubbed for 5 min. in a mortar and the tissue washed into centrifuge tubes with 100 ml. of 0·9% NaCl, leaving behind the bulk of the sand. After centrifuging, the supernatant was discarded and the precipitate suspended in a further 100 ml. of NaCl. In most instances reported here such washing was performed six times in all, although the preparations did not noticeably change after one or two washings (see p. 472). Preparations were stored at 2–4° as suspensions containing tissue from 1 g. fresh weight of brain in 2 ml. of 0·9% NaCl saturated with toluene. Metabolic quotients were calculated from the dry weights of the whole tissue from which the preparations had been made.

Determination of nicotinic acid and nicotinamide

The reaction with cyanogen bromide (see especially Teeri & Shimer, 1944) has been adapted to the special problems associated with the determination of nicotinamide in the presence of cozymase.

Reagents. The phosphate buffer was that of Martinek, Kirch & Webster (1943). CNBr solution was prepared from sat. Br_2-water (500 ml.) and KCN (10% w/v, 70–80 ml.) at 5–10° and kept in a refrigerator for not more than a week. The necessary concentration of HCl used was found to be approx. 5 N for nicotinic acid and N for nicotinamide.

Nicotinic acid. To the CNBr (2·5 ml.) and buffer (5 ml.) solutions in glass-stoppered tubes, the specimens (containing up to 0·5 μmol. nicotinic acid in 5 ml.) were added and the mixtures left at room temperature for 35 min., *m*-phenylenediamine dihydrochloride (5% in HCl, 0·5 ml.) and HCl (0·5 ml. 5 N) were added rapidly, mixing after each addition. The intensities of the resulting yellow colours were determined within 30 min. in a Spekker absorptiometer with Ilford spectrum filter no. 601. A reagent blank (water substituted for the test solution) was subtracted.

Nicotinamide. The amide yielded about a quarter of the colour intensity afforded by the acid under the above conditions (cf. Melnick & Field, 1940; Perlzweig, 1947). Several workers have used heat during the reaction with CNBr to reduce the reaction time and increase the colour intensity (Bandier & Hald, 1939; Martinek *et al.* 1943); in the presence of cozymase we found most satisfactory the following modification of the method described above for nicotinic acid. The mixtures of CNBr, buffer and specimens (up to 0·5 μmol. nicotinamide) were kept at 37° for 30 min., cooled to approx. 10°, the amine (0·5 ml.) and HCl (0·5 ml. N) added, and intensities determined within 40 min.

Preparation of specimens for analysis. Specimens were mainly from reaction mixtures containing brain tissue or preparations, and it was necessary to deproteinize them without liberating nicotinamide from cozymase. $Zn(OH)_2$ was found satisfactory. To the specimen (containing approx. 5–40 mg. wet wt. of tissue) in a graduated centrifuge tube $ZnSO_4$ (8% w/v, 0·2–0·4 ml.) was added and the tube put in ice water. NaOH solution, equal in volume to the $ZnSO_4$, and of a concentration previously found sufficient to remove the Zn and leave a neutral solution, was added followed by water to 7 or 10 ml. The tubes were centrifuged and samples taken for nicotinamide determination.

Acid formation during cozymase breakdown

In the main compartments of Warburg vessels (conical, of approx. 15 ml.) were placed 2 ml. of a solution containing $NaHCO_3$ (0·009 M) and NaCl (0·9%), freshly gassed with

5% (v/v) CO_2-N_2. To the side arms were next added 0·5 ml. of the bicarbonate saline (controls) or cozymase freshly dissolved in bicarbonate saline (experimental vessels). The tissue suspension (0·5 ml., in 0·9% NaCl) was then added to the main compartments, yellow P to the centre cups, the vessels fitted to manometers and equilibrated with the CO_2-N_2 at 37°. Successive readings were usually steady 15–20 min. after placing in the thermostat; the contents were then mixed and readings taken at 3 or 5 min. intervals.

Other physiological salines used were those of Krebs & Eggleston (1940).

RESULTS

Rate of reaction

Estimation by nicotinamide formation. First indications of the high reaction velocity were obtained by following the course of liberation of nicotinamide by brain tissue ground with sand (Fig. 1). Cozymase concentrations were employed which approximated to those of the pyridine nucleotide content of animal tissues. After stopping the reaction and removing protein (both accomplished by zinc hydroxide precipitation) nicotinamide was determined by the Koenig reaction. The initial velocity in Fig. 1 corresponds to the decomposition of 1·8 μmol. of cozymase/mg. dry wt. of whole tissue/hr. Other experiments by this method under conditions similar to those of Fig. 1 gave quotients of 1·0–1·4 with tissue from different species (Table 2).

Direct comparison between the rate of breakdown of cozymase and the rate of respiration was made with material from guinea pig brain. Slices (mixed grey and white matter from cerebral hemispheres) absorbed 0·64, 0·60 μmol. O_2/mg. dry wt./hr. in glucose saline, without Ca salts, at pH 7·4 and at 37° (O_2 as gas phase). Values in saline with Ca (0·005M) were 0·4, 0·38 μmol. O_2/mg. dry wt./hr. These are typical values (Warburg, 1924; Quastel, 1939; Elliott & Libet, 1942). Ground tissue from the same hemispheres decomposed 1·25, 1·35 μmol. cozymase/mg. dry wt./hr.

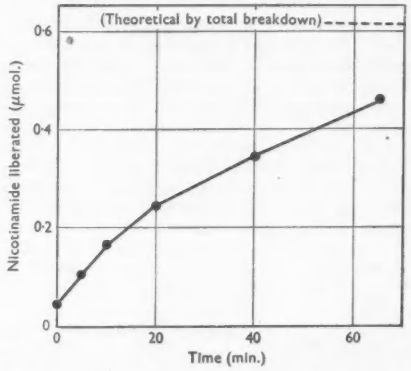


Fig. 1. Course of liberation of nicotinamide from cozymase (0·62 μmol.) by ground rabbit brain (0·4 mg. dry wt.) in 3·2 ml. phosphate Ringer at pH 7·4; 37°; aerobic.

Activity of different tissue preparations. The rate of reaction increased with the degree of damage of the tissue (Table 2), being less in slices or crushed tissue

Table 2. *Cozymase breakdown by different species and preparations*

Animal	Tissue	Treatment	Rate of breakdown (μmol./mg. dry wt./hr.)
Rabbit	Whole brain	Ground with sand	1·4
Guinea pig A	,,	,,	1·0, 1·4
Sheep A	,,	,,	1·3
Mouse	,,	,,	0·5
Sheep B	Cord	Homogenized	1·08
	White matter from corpus callosum	,,	0·86
	Grey matter from lateral convexity of hemispheres	,,	0·36
	Grey matter adjoining longitudinal fissure	,,	0·36
Guinea pig B	Whole cerebral hemisphere	Ground with sand; not washed	1·02
	,, ,,	Ground with sand; washed 3 times	1·03
	,, ,,	Homogenized in equal weight of saline	1·15
	,, ,,	Homogenized in × 20 wt. of saline	1·12
Guinea pig C	Largely white matter from centrum ovalae	Sliced	0·28, 0·46
	Largely grey matter from cerebral cortex	,,	0·13
Guinea pig D	Largely corpus callosum	Teased	0·43
	Mixed tissue from cerebral hemispheres	Crushed	0·51
	,, ,, ,, ,,	Homogenized	1·56

than in homogenates or material ground with sand. In grinding with sand, little difference was found between a preparation rubbed for 0·5 and one rubbed for 5 min. No appreciable difference was found between the activity of a suspension of guinea pig brain made 2–3 min. after death, and another made after the excised tissue had been left at room temperature for 30 min. White matter was more active than grey (Table 2).

Acid formation during the reaction

The findings of Handler & Klein (1942) on the breakdown of cozymase suggested to us that the end products might be more acidic than cozymase, since the reaction involves the conversion of a pyridinium salt, the salt of a strong base, to a much more weakly basic pyridine derivative (see equation I, p. 474). The breakdown of cozymase has been followed in previous work either by measuring the fall in catalytic activity of cozymase-containing solutions or by measuring the nicotinamide produced. Measurement by acid formation would be much more convenient in studying the reaction kinetically, but it was first necessary to find whether the same breakdown was in fact being observed. Relevant findings are given below.

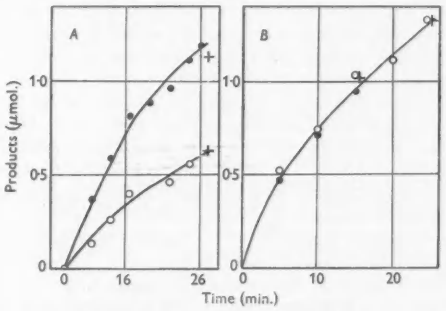


Fig. 2. Course of evolution of CO_2 from $NaHCO_3$-containing solutions during breakdown of cozymase by ground brain (points); relationship to nicotinamide released (crosses). (*A*) Rabbit; pH, 7·3, maintained by $NaHCO_3$ (0·021 M) and CO_2-N_2 (5% v/v). Reaction mixtures contained also 0·9% NaCl, 1·24 μmol. cozymase and the following dry weights of tissue: —○—○—, 1 mg.; —●—●—, 2·5 mg. (*B*) Sheep; pH 6·8 (by $NaHCO_3$-CO_2); cozymase, 2·4 μmol., 4·2 mg. dry weight of tissue; otherwise as (*A*). The two sets of points (○ and ●) refer to duplicate reaction mixtures. Crosses indicate nicotinamide released at the end of each experiment.

Quantity of acid produced. By carrying out the reaction in bicarbonate-containing solutions in manometric apparatus it was possible to observe a smooth evolution of CO_2 (Fig. 2). The use of such conditions for measuring the rate of the reaction was, therefore, examined. Anaerobic conditions were generally employed, to minimize the possibility of associated reactions. The brain preparations after three washings with 0·9% sodium chloride caused very little gas change at the low concentrations in which they were used (0·5–2 mg. dry wt./ml.; 3 ml./vessel). Reaction in the absence of cozymase was relatively very small even when fresh homogenates of tissue were used without any washing. The commonest observation was of gas absorption in the absence of substrate, at a rate between 0 and 10% of the rate of reaction with cozymase. Such changes were allowed for by subtracting the change in a tissue control from those in experimental vessels. Any retention of CO_2 by the preparations was within experimental error. Using such methods, the CO_2 evolved was found to be approximately equivalent to the cozymase decomposed (Fig. 2). Seventeen experiments with five different preparations, including sheep, rabbit and guinea pig brain, gave for the ratio: CO_2 evolved/nicotinamide formed, a mean value of 0·98 with standard deviation of 0·08.

With limited quantities of cozymase the gas evolution reached a well-marked end point equivalent to the cozymase added. At this stage the enzyme was not inactive; this was shown by addition of further cozymase. The end point is being approached in one experiment of Fig. 2*A*.

Inhibition by nicotinamide. Mann & Quastel (1941) found 0·1–0·01 M-nicotinamide prevented the inactivation of cozymase by animal tissues, including brain. This finding has been applied by many subsequent workers in experiments which required

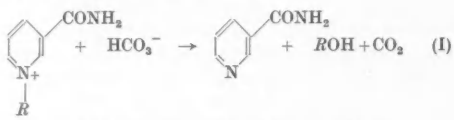
Table 3. *Inhibition of the breakdown of cozymase by nicotinamide*

(Reaction mixtures contained a sheep-brain preparation (1·7 mg. dry wt.) and the nicotinamide in 2·5 ml. NaCl-$NaHCO_3$. Cozymase (in 0·5 ml.) was added at zero time, and the reaction run for 26 min. The rates of the last column were determined graphically.)

In reaction mixture		Produced		Initial rate of CO_2 evolution (μmol./mg. dry wt./hr.)
Cozymase (μmol.)	Nicotinamide (μmol.)	Nicotinamide (μmol.)	CO_2 (μmol.)	
1·24	0	1·05	1·01	1·26
1·24	1·0	0·92	0·96	1·13
1·24	2·0	0·82	0·78	0·75
1·24	4·0	0·60	0·55	0·56
1·24	8·0	—	0·26	0·34

intact cozymase, relatively high concentrations of the amide (e.g. 0·02 M) being employed. We found both 0·01 and 0·002 M-nicotinamide almost completely inhibited the formation of acid from cozymase by washed sheep brain, under the experimental conditions described above. This additional evidence supports the validity of the manometric method for

following cozymase breakdown. Moreover, the reaction was found to be extremely sensitive to nicotinamide. A quantity equimolar to the cozymase content of a typical reaction mixture markedly lowered the reaction rate (Table 3). Because of this sensitivity it was possible to follow the inhibited reaction quantitatively not only by acid formation but also by nicotinamide production. Good agreement was obtained between measurements made in these two ways (Table 3). This makes it probable that the acid and nicotinamide are indeed formed in the same reaction, and, accepting the evidence of Handler & Klein (1942), this may be formulated as in (I), though we have not fully investigated alternative possibilities.



(I)

Rate of cozymase breakdown under various conditions

The effect of a variety of circumstances on the breakdown was examined. In doing this the rate of acid formation was largely used, but results were confirmed in each set of experiments by measuring at the end of the reaction the total nicotinamide formed.

pH, *salts, oxygen.* The reaction showed an optimum pH between 6·5 and 7 (Fig. 3). Most manometric experiments were, therefore, carried out at pH 6·9,

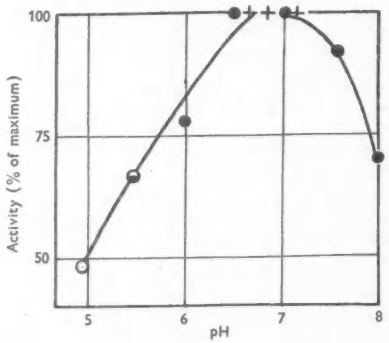


Fig. 3. Change in rate of cozymase breakdown with pH. ●, 0·02 M-phosphate buffers, guinea pig brain; ○, 0·1 M-acetate buffers, guinea pig brain; +, bicarbonate-5% v/v CO_2, rabbit brain. Initial concentration of cozymase 2×10^{-4} M.

obtained by 0·0075 M-sodium bicarbonate in equilibrium with 5% (v/v) CO_2-N_2. Great variation in the ionic composition of the medium was possible without significant change in the reaction velocity (Table 4 and Fig. 3). The reaction velocity was compared in air/CO_2 and N_2/CO_2 (with yellow phosphorus to maintain anaerobiosis), and no difference was found.

Table 4. *Cozymase breakdown in different media*

(All mixtures contained cozymase (2×10^{-4} M) and were at pH 7·4. Each of the velocities quoted was calculated from three determinations of the nicotinamide liberated at different times during the reaction.)

Suspending medium	Rate of breakdown (μmol. nicotinamide liberated/mg. dry wt./hr.) With guinea pig brain	With rabbit brain
Physiological saline with Ca	1·07	1·26
Physiological saline without Ca	1·17	1·25
Phosphate buffer (0·02 M)	0·96	1·17

Stability of preparations. Ground sheep brain, washed and suspended (4·4 mg. dry wt./ml.) in 0·9% sodium chloride at pH 7·0, retained less than 5% of its activity after heating for 15 min. at 100 or 70°. At 60°, 20% of the activity remained and at 50°, 40%. At 37°, no loss occurred in 1 hr. at pH values (in phosphate) of 5·6, 6·8 and 8. Preparations were normally stored in a refrigerator at 2–3°, with 0·5 g. wet wt. of tissue/ml. of 0·9% sodium chloride. Most preparations had been washed with 0·9% sodium chloride (volume, five times the bulk of fresh tissue) by centrifuging either three or six times, and were, without deliberate adjustment of pH, at pH 7. Toluene was added as a preservative and under these conditions little loss in cozymase-splitting activity occurred during 1–2 months. Some loss was found in preparations 3 or 4 months old, and in a more recent preparation in which bacteria had grown.

Variation in velocity with cozymase concentration; different cozymase specimens. Figs. 1 and 2 show that the velocity of breakdown of cozymase falls towards the end of experiments in which cozymase concentration is decreasing, and the concentration of nicotinamide, which inhibits the reaction, is increasing. To differentiate between these two factors, kinetic experiments were carried out with different initial concentrations of cozymase, and initial velocities of breakdown obtained from graphs such as those of Fig. 2. The initial velocity fell relatively little at concentrations down to 6×10^{-4} M, the lowest at which the ordinary manometric method is applicable. The reaction velocity at concentrations below this was, therefore, followed by nicotinamide determinations. The results (Fig. 4) were not sufficiently accurate for determining the equilibrium constants of the system, but showed that the velocity did not fall to half the maximum until the concentration of cozymase was less than 10^{-4} M. A higher value is given by Spaulding & Graham (1947) for unspecified

tissues which may include brain. Rates of breakdown of specimens A, D and E of cozymase (see Table 1) were compared at concentrations between 1·3 and 4×10^{-4} M; no differences were found.

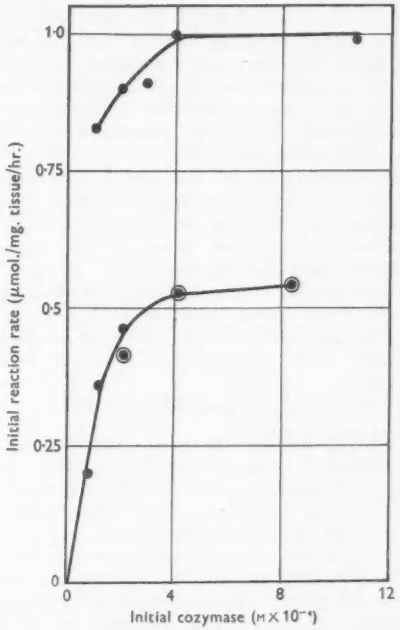


Fig. 4. Change in initial rate of cozymase breakdown with cozymase concentration, in two preparations of guinea pig brain. Above: cozymase E; below, cozymase D. ⊙, determined by CO_2 evolution; ●, by nicotinamide formation.

Extraction of cozymase-splitting enzyme

In the preceding experiments a suspension of tissue particles in 0·9 % sodium chloride was used in which the activity was firmly associated with the particles (Table 2). The active material readily dissolves in water with little loss, together with a large part of the tissue preparation, giving an opalescent solution; as is shown in Table 5 the activity/unit dry weight is increased in the process. Sheep brain has been extracted similarly, both before and after standing with toluene. The properties and treatment of such extracts will be described later.

DISCUSSION

The following findings must be taken into account in assessing the part played by the breakdown of cozymase in preparations from nervous tissue. (1) Its velocity of 0·4–1·8 μmol./mg. dry wt./hr. This is outstanding. The rate of reaction, even on a molar basis, can be greater than the respiratory rate of the tissue; it can lead to the breakdown of the tissue's own dry weight of cozymase each hour. (2) This velocity is reached at the concentrations of cozymase occurring in the tissue. (3) The reaction is inhibited by nicotinamide, also at concentrations comparable to those at which nicotinamide derivatives occur in the tissue. It is (4) most rapid in damaged tissue, leads (5) to loss of the catalytic activity of cozymase and (6) to the formation of hydrogen ions at an equivalent, high velocity.

Table 5. *Extraction of cozymase-splitting system from guinea pig brain*

Preparation	Activity (by CO_2 evolution at pH 6·8): μmol. cozymase split/material derived from 1 g. fresh wt./hr.	Activity (by CO_2 evolution at pH 6·8): μmol. cozymase split/mg. dry wt. of preparation/hr.
(1) Whole brain ground with sand	224	1·12
(2) Preparation (1) washed with saline and extracted with water (5 ml./g. fresh brain); extract	77	1·6
(3) Residue from (2) re-extracted as before; extract	77	1·4
(4) Residue from (3)	58	0·6
Sum of (2), (3) and (4)	212	—

Reaction velocity. Although the reaction has been investigated in ground nervous tissue and has frequently been described as rapid, kinetic experiments leading to dependable values for its rate do not appear to have been reported. An isolated statement is made by Spaulding & Graham (1947, in a footnote to Table 1), but not discussed further, that 'the nucleosidase/g. of dry brain...split approximately 15 mM of DPN in 20 min.' Interpreting mM as millimol., the velocity corresponds to 100 times that which we find; on the other hand, most other measurements of cozymase in Spaulding & Graham's paper are in micromol. quantities, and if mM means micromol. the speeds are about 1/10 those which we find. Spaulding & Graham give the relative velocities of breakdown by ground rat brain and heart as in the ratio 100:27. The rate of breakdown of cozymase by guinea pig heart can be derived indirectly from measurements by Govier & Jetter (1948) as about 0·3 μmol./mg. dry wt./hr.; ignoring possible species differences, this would suggest a value of about 1·1 μmol./mg./hr. for brain. This is close to our own findings and appears to be the most satisfactory interpretation of the observations of Spaulding & Graham (1947). We have preferred not to use a name

such as diphosphopyridine nucleotidase for the system, as the specificity of its action has not yet been examined.

Dependence on nicotinamide and cozymase concentrations. The concentration of cozymase in fresh brain tissue can be calculated from values given by Axelrod, Madden & Elvehjem (1939) as approx. 5×10^{-4}M. Values for total nicotinic acid derivatives are close to this (Mitchell & Isbell, 1942; Taylor, Pollack & Williams, 1942). With such an initial concentration of cozymase, our results show that the breakdown can proceed at over 80% of its maximum velocity, but that a fall in cozymase concentration results in a fall in the velocity of the reaction (Fig. 4). Also, the reaction is inhibited by comparable concentrations of nicotinamide, one of its products. Therefore, although the operation of this reaction at its maximum velocity would lead to very rapid depletion of the cozymase of the tissue, the velocity of breakdown would be expected to fall considerably as the substrate disappeared and the product accumulated.

Possible operation of the reaction in vivo. Although the breakdown of cozymase is particularly rapid in brain tissue, and may there subserve a special purpose, it occurs in many animal tissues (cf. Schlenk, 1945; Spaulding & Graham, 1947; Govier & Letter, 1948) and also in bacteria (McIlwain & Hughes, 1948). Data are only rarely available for full characterization of the reaction, but it is readily differentiated from that of the nucleotide pyrophosphatase (Kornberg, 1948) which does not liberate free nicotinamide. The process in streptococci liberated nicotinamide, but was not associated with acid formation (McIlwain & Hughes, 1948); there, however, intact cells were being examined, and greater possibility existed for coupling with further changes. The major problem in assessing the physiological importance of the reaction lies in reconciling its high velocity with the undoubted importance of intact cozymase. In general, this suggests that the breakdown may be intermittent, owing to some physiologically controlled inhibition; that the breakdown must be counterbalanced by synthesis; and that the reaction may constitute part of a system controlling cozymase-requiring reactions by regulating the cozymase level.

Considering more specifically mammalian brain, the cozymase content of the fresh organ in a given species has been found to be relatively constant (Axelrod *et al.* 1939), and breakdown of added cozymase is smallest when least damage has been done to the tissue. In the tissue, which of course contains more than one cell type, the process is presumably inhibited for a large part of the time (possibly by separation of the reactants), and balanced by resynthesis if and when it takes place. Stability of the native cozymase of the tissue is being examined separately, but it is possible to make the following observations from the present data. The reaction seems suited to physiological operation and control in that its pH optimum is within physiological range; its relation to substrate concentration is such that with the cozymase concentration found in the tissue as a whole the reaction would be rapid initially, but decrease in rate as cozymase is lost and nicotinamide accumulates; the high initial velocity makes it less likely that reaction with cozymase is an incidental property of a system whose main reaction is with another substrate. The phenomena of functional importance in nervous tissue are intermittent, extremely rapid and associated with ion migration and permeability changes. These give many possibilities for the functional importance of a reaction with the characteristics of the cozymase breakdown, but more data are required for their detailed discussion.

SUMMARY

1. The process by which tissue preparations from the central nervous system liberate nicotinamide from cozymase proceeded at rates of 0·4–1·8 μmol./mg. dry wt./hr.; this was often greater than the rate of respiration by the tissue.

2. The reaction approached its maximum velocity at the natural concentration of cozymase in brain tissue (5×10^{-4}M); half the maximum velocity was reached at less than 10^{-4}M-cozymase.

3. The breakdown was very sensitive to nicotinamide; 10^{-3}M-nicotinamide reduced the rate of breakdown of 3×10^{-4}M-cozymase to half its uninhibited value.

4. The process resulted in the formation of one equivalent of acid/molecule of nicotinamide liberated. This was apparently due to the disappearance of the pyridinium ion, and when the reaction was carried out in $NaHCO_3$-CO_2 buffers its velocity could readily be followed manometrically by CO_2 evolution.

5. The optimum pH of the reaction was approx. 7, and the velocity was almost unaffected by many inorganic salts.

6. The system responsible for the reaction remained associated with tissue debris in isotonic solutions, and was fairly stable at 0 or 37°, but inactivated rapidly at 70°. It was easily obtained in solution in water.

We are indebted to Mr J. D. Cheshire for assistance during these experiments.

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The Oxidation of Catechol and Homocatechol by Tyrosinase in the Presence of Amino-acids

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In the early stages of the oxidation of catechol by crude extracts of potato or mushroom the formation of reddish purple pigment can be detected. Attention was drawn to this phenomenon first by Szent-Györgyi (1925), who achieved some degree of separation from potato juice of the substance which, together with enzyme and catechol, was responsible for the pigment formation. He suggested the name 'tyrin' for it and speculated as to its possible significance as a reversibly oxidizable hydrogen carrier. Platt & Wormall (1927), however, subsequently concluded that 'tyrin' was merely a mixture of amino-acids, and no more has been heard of it. Although, as Platt & Wormall believed, many amino-acids can cause pigment formation when present in solutions in which tyrosinase is oxidizing catechol, there is now reason to think that the amino-acid contributing most strikingly to the phenomenon originally observed is L-proline. The identification of L-proline as a constituent of mushroom extract, and the results of a study of some features of the reaction involved in the production of the pigment, are described in this paper.

METHODS

Tyrosinase preparations. These were made from the common mushroom (*Psalliota campestris*) according to the directions of Keilin & Mann (1938), but with the omission of some of the later steps in the purification described by these workers. Various preparations with Q_{O_2} values ranging between 50,000 and 500,000,* were used in the course of the work. Within these limits variation in the purity of the enzyme preparation has no qualitative effect on the reactions which are to be discussed.

Measurement of oxygen uptake. The Warburg manometric apparatus was used. The total volume of reaction mixture was 2·0 ml., the components being distributed in 0·1 M-phosphate buffer of pH 7·0. The enzyme was tipped into the substrate solution from the side arm at zero time, and in critical experiments the inset cup of the reaction vessel contained 20% KOH and a roll of filter paper. In the earliest experiments the temperature of the water bath was 20°, but was changed to 25° when the advent of warmer weather made the maintenance of the lower temperature difficult.

Colorimetry. In experiments where quantitative assessment of pigment formation was attempted a Spekker photoelectric absorptiometer was used with a 1 cm. cell and a green filter (no. 5 of Hilger set H 455, later replaced by Ilford spectrum green no. 604). The destruction of the pigment in acid solution was similarly studied, readings being taken at timed intervals after addition of acid to the pigment solution.

Measurement of pH *and hydrogen-ion liberation.* A glass electrode was used for pH measurement. When it was desired to study the hydrogen-ion liberation accompanying the oxidation of catechol in the presence of amino-acid the reaction was carried out in unbuffered solution at pH 7·0.

* Q_{O_2} values in μl./mg. dry wt./hr., substrate catechol, determined manometrically according to Keilin & Mann (1938).

A glass electrode was immersed in the reaction mixture, which was aerated with CO_2-free air or O_2. Standard NaOH was added from a microburette as necessary to maintain the pH at its initial value. The amount of alkali added was then the measure of the hydrogen ion liberated during the reaction.

RESULTS

Preliminary attempts to isolate from mushroom extract the substance responsible for the pigment formation suggested that this substance might be an amino-acid. Of the available pure amino-acids tested in the tyrosinase-catechol system, only proline and hydroxyproline gave a colour similar to that given by the mushroom concentrates. That one of these acids might be concerned was further suggested by the fact that the more active the concentrate, the lower was the ratio of amino nitrogen to total nitrogen in it. When *p*-benzoquinone was shaken with proline in ethanol a very similar colour was produced suggesting that the pigment formation in the enzyme reaction was the result of a secondary reaction of amino-acid with *o*-benzoquinone, analogous to anilinoquinone formation in the presence of aniline (Pugh & Raper, 1927).

Isolation of L-*proline from mushrooms*

The mushrooms were minced into 95% ethanol, and after standing 2–3 hr., with frequent stirring, the supernatant fluid was decanted and the tissue squeezed out in muslin in a hand press. After filtration of the combined fluids the ethanol was removed under reduced pressure and the residue taken up in water. Sufficient basic lead acetate was added to ensure maximum precipitation, and the precipitate was removed by filtration. After removal of the lead by H_2S the filtrate was taken to dryness at 30° *in vacuo* and extracted with cold methanol. The methanol was removed from the extract under reduced pressure, and the residue dissolved in water and precipitated with Reinecke salt. This precipitation was carried out in two stages, separating first the precipitate formed at 25°, and then lowering the temperature to 5°, when a second crop of Reineckate was obtained. The precipitates were decomposed separately by the method of Kapfhammer & Eck (1927), and the solutions were evaporated to dryness under reduced pressure. The material from the Reineckate precipitate obtained at 5° gave a white crystalline solid on treatment with absolute ethanol. Recrystallization from absolute ethanol gave a product of m.p. 218–220° and $[\alpha]_D^{20°}$ – 76° in water. The figures usually quoted for L-proline are m.p. 220–222° (Kossel & Dakin, 1904), and $[\alpha]_D^{20°}$ – 80° (Fischer & Zemplen, 1909). (Found: C, 52·3; H, 8·0; N, 12·0. Calc. for proline $C_5H_9O_2N$: C, 52·2; H, 7·8; N, 12·2 and for hydroxyproline $C_5H_9O_3N$: C, 45·8; H, 6·9; N, 10·7%.) It is concluded therefore that the product was L-proline. About 1 g. was obtained from 7 kg. of mushrooms.

Oxidation of catechol by tyrosinase in the presence of proline

Fig. 1 shows the course of O_2 uptake when a small amount of catechol was oxidized by excess of tyrosinase under the conditions already specified, in the presence of different molar proportions of DL-proline. No special significance is to be attached to the initial rates of uptake, which were limited by the rate of diffusion of oxygen into the liquid phase, but it is clear that in the presence of proline the uptake of the second atom of oxygen/mol. of catechol was greatly accelerated. Provided a sufficient amount of proline was present, the absorption of oxygen was completed within 10 min., and the amount then absorbed corresponds within the limits of experimental error to 2 atoms/mol. of catechol. The intense purple end product appeared to undergo no further change during the time of the experiment.

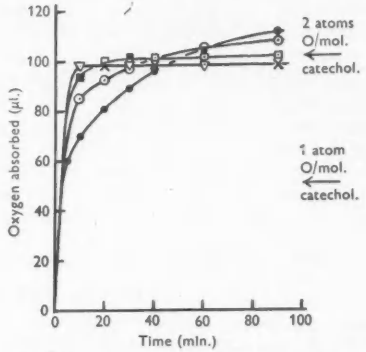


Fig. 1. Oxidation of 0·5 mg. catechol by tyrosinase in the presence of proline, at pH 7·0 and 20°. Mol. proline present/mol. catechol: ●—●, none; ⊙—⊙, 0·5; ⊡—⊡, 1·0; ▽—▽, 2·0; ×—×, 5·0.

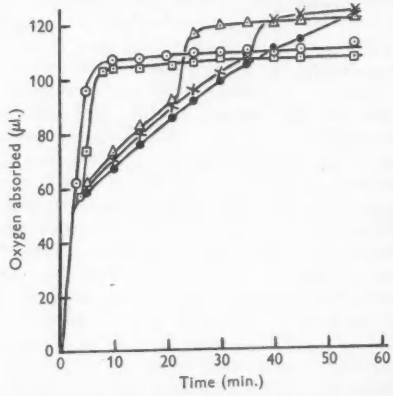


Fig. 2. Oxidation of 2·0 ml. 0·0025 M-catechol by tyrosinase in the presence of 0·025 M-hydroxyproline at pH 6·0 and 25°. The hydroxyproline was introduced into the reaction mixture ⊙—⊙, before the beginning of the oxidation; ⊡—⊡, at 4 min.; △—△, at 22 min.; ×—×, at 36 min.; and ●—●, not at all.

When hydroxyproline is substituted for proline in such an experiment the course of O_2 uptake and the colour developed are scarcely distinguishable from those observed in the case of proline. Fig. 2 shows the O_2 uptakes in an experiment in

which the addition of hydroxyproline to the reaction mixtures was delayed for a period after the enzymic oxidation of catechol had begun. Warburg flasks with two side arms were used, the second side arm containing the imino-acid solution which was tipped into the main part when the desired time interval had elapsed. The experiment was carried out at pH 6·0 in order to slow down the absorption of the second atom of oxygen. It will be noted that the addition of the imino acid caused in each case a rapid absorption the magnitude of which diminished with increasing delay in imino acid addition; but the total uptake at the end of the experiment was the smaller, and the nearer to precisely 2 atoms/mol. catechol, the earlier the imino acid addition. At the end of this experiment each reaction mixture was diluted to 50 ml. and its colour intensity measured in the Spekker absorptiometer. The results obtained are shown in Table 1. Little reduction in pigment formation was observed

Table 1. *Effect of delay in hydroxyproline addition on pigment formation in the catechol-tyrosinase system*

(Reaction mixture 2·0 ml., pH 6·0, 25°. 0·0025 M-catechol, 0·025 M-hydroxyproline. Diluted with water to 50 ml. for absorptiometer reading.)

Time after beginning of oxidation when hydroxyproline was added (min.)	Atoms of O/mol. catechol already absorbed at this time	Final colour intensity*
0	0	0·490
4	1·03	0·430
22	1·66	0·279
36	1·95	0·217
No addition	—	0·107

* Absorptiometer reading, filter no. 604.

when the addition of hydroxyproline was delayed until 1 atom of oxygen/mol. catechol had been absorbed, but the uptake of the second atom of oxygen was accompanied by a corresponding loss of capacity for pigment production upon addition of imino acid. Similar results were recorded by Jackson (1939) in a study of the oxidation of catechol in the presence of aniline, and were interpreted as indicating that only the primary oxidation product of catechol, i.e. *o*-benzoquinone, reacted with the base to form pigment. The same interpretation fits the facts of the present case.

Oxidation of catechol by tyrosinase in the presence of glycine

The effect of glycine on the oxidation is shown in Fig. 3. In this case, too, the initial very rapid O_2 uptake was greater in the presence of the amino-acid. At the lower glycine concentrations the absorption had almost ceased in 50 min., and the total amount absorbed at the end of the experiment was less than in the absence of amino-acid, though significantly in excess of 2 atoms/mol. catechol. At the highest glycine concentration the rapid phase was followed by a further O_2 uptake which continued at a considerable and almost constant rate until the end of the experiment. The colour developed is an orange pink, and the observations suggest that a fairly stable pigment is formed when catechol and glycine are in equimolar proportions, and that when glycine is present in excess oxidation of the excess takes place, as indicated by the continued O_2 uptake. This oxidation is accompanied by liberation of NH_3, as was observed by Platt & Wormall (1927).

A few similar experiments were made with alanine, glutamic acid and arginine, with similar results. In each case, at relatively high amino-acid concentrations, the rapid phase of O_2 uptake ending at about 2 atoms/mol. catechol was followed by a slower continued absorption indicative of oxidation of the amino-acid. The rapidity of this oxidation diminished in the series glycine, arginine, glutamic acid, alanine.

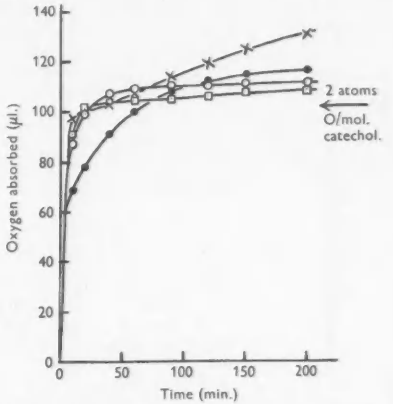


Fig. 3. Oxidation of 0·5 mg. catechol by tyrosinase in the presence of glycine, at pH 7·0 and 20°. Mol. glycine present/mol. catechol: ●—●, none; ⊙—⊙, 1·0; ⊡—⊡, 2·0; ×—×, 5·0.

Oxidation of catechol by tyrosinase in the presence of pyrrolidine

It is of interest to know whether pyrrolidine, like its derivatives proline and hydroxyproline, gives rise to pigment formation and affects the O_2 uptake in the tyrosinase-catechol system. Tests showed pyrrolidine to behave like its carboxylated derivatives. The effect of pyrrolidine on the O_2 uptake is seen in Fig. 4, where curves relating to similar experiments with hydroxyproline and with aniline are given for comparison.

Oxidation of homocatechol by tyrosinase in the presence of amino-acids

Pigment formation from homocatechol (4-methylcatechol) took place in a manner exactly analogous, so far as could be judged, to its formation from catechol. In Fig. 5 the O_2 uptakes of catechol and homocatechol under the same conditions with and without proline are compared. The reaction rate, particularly as regards the uptake of the second atom of oxygen, was slower in the case of homocatechol, but the amino-acid exerted a similar effect with each substrate. When 4:5-dimethylcatechol was present as substrate, the addition of proline had no effect on the colour which developed in the reaction mixture.

Pigment formation from monophenols and imino acids

By virtue of their monophenolase activity tyrosinase preparations are able to form pigment from phenol and *p*-cresol. The pigments formed in the presence of the imino acids appear to be identical with those formed from catechol and homocatechol respectively. The course of O_2 uptake with monophenol as substrate to the enzyme is shown in Fig. 6, and is seen to be more rapid in the presence of hydroxyproline. The total uptake is about the expected 3 atoms/mol. monophenol. The induction period before the O_2 absorption rate reaches its maximum, a well-known characteristic of the oxidation of monophenol by the enzyme, is discernible in the phenol curves, though at the high enzyme concentration used in the experiment it was very brief. It is of interest to note that the duration of this period is much diminished by the presence of the imino acid, an effect which is seen more clearly in experiments with smaller enzyme concentrations.

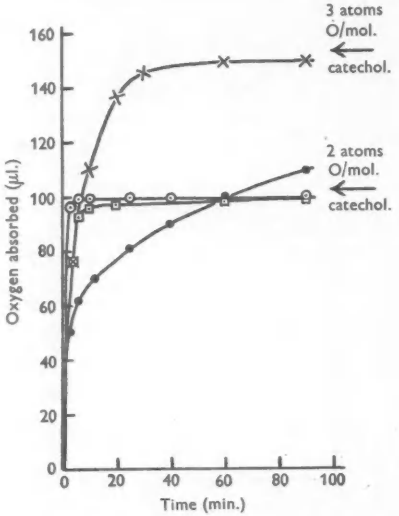


Fig. 4. Oxidation of 0·5 mg. catechol by tyrosinase at pH 7·0 and 20°, alone (●—●) and in the presence of excess hydroxyproline (⊙—⊙), pyrrolidine (⊡—⊡) and aniline (×—×).

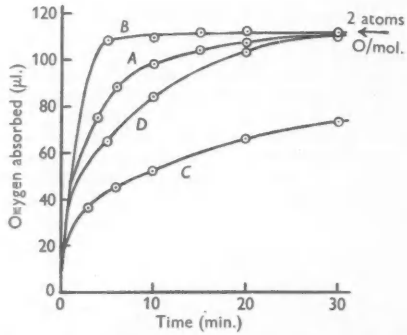


Fig. 5. Oxidation of 2·0 ml. 0·0025 M-*o*-diphenols by tyrosinase at pH 7·0 and 25°, alone and in the presence of 0·025 M-proline. *A*, catechol; *B*, catechol and proline; *C*, homocatechol; *D*, homocatechol and proline.

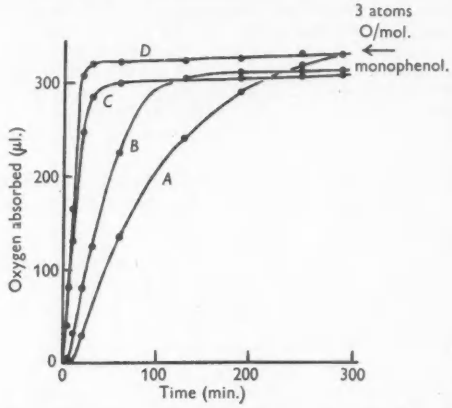


Fig. 6. Oxidation of 2·0 ml. 0·005 M-monophenol by tyrosinase at pH 7·0 and 25°, with and without 0·01 M-hydroxyproline. *A*, phenol; *B*, phenol and hydroxyproline; *C*, *p*-cresol; *D*, *p*-cresol and hydroxyproline.

In an experiment similar to that to which Fig. 6 refers, but including in addition reaction mixtures with equivalent concentrations of catechol and homocatechol instead of monophenol, the pigmented reaction mixtures at the end of the manometric experiment were equally diluted, and an estimate of relative pigment concentrations obtained by taking absorptiometer readings. The readings for the products from phenol and catechol were 0·690 and 0·678 respectively, for those from *p*-cresol and homocatechol 0·546 and 0·460. Thus pigment formation was with the former pair slightly, with the latter pair markedly, more efficient when the substrate was monophenol than when it was the corresponding *o*-diphenol.

Colorimetric study of pigment formation

To obtain information about the relationship between the extent of pigment formation and the molar ratio of imino acid to catechol during the reaction, mixtures containing 5 µmol. catechol and excess enzyme, with varying proportions of imino acid, at pH 7 and 25°, were shaken with air long enough to ensure maximum colour development, then diluted to 25 ml. with water and examined in the Spekker absorptiometer with the Ilford filter no. 604. This procedure was also carried out with homocatechol, and colour development with pyrrolidine, hydroxyproline ethyl ester, glycine, dimethylamine and methylamine, as well as with proline and hydroxyproline, was examined. The results are shown in Figs. 7 (*a*) (catechol) and 7 (*b*) (homocatechol), where the

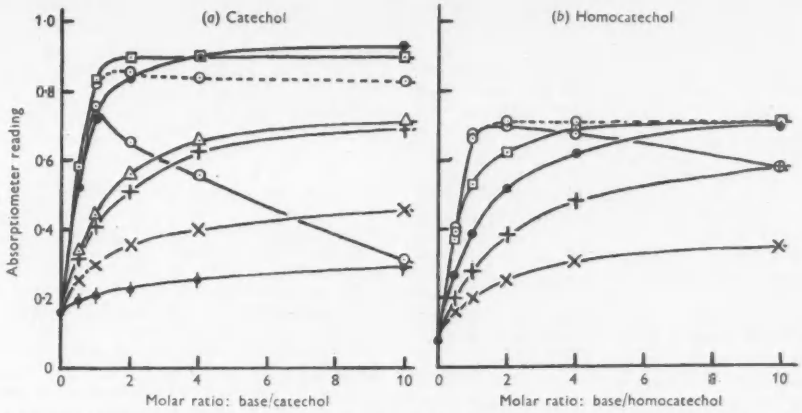


Fig. 7. Effect of variation in concentration of nitrogenous base on the colour developed during the oxidation of *o*-diphenols by tyrosinase at pH 7·0 and 25°. (*a*), catechol; (*b*), homocatechol. The broken line represents the results of an experiment in which the pH was 6·0. The bases used were ●—●, proline; ⊡—⊡, hydroxyproline; ⊙—⊙, hydroxyproline ethyl ester; +—+, pyrrolidine; ×—×, glycine; △—△, dimethylamine; ⧫—⧫, methylamine.

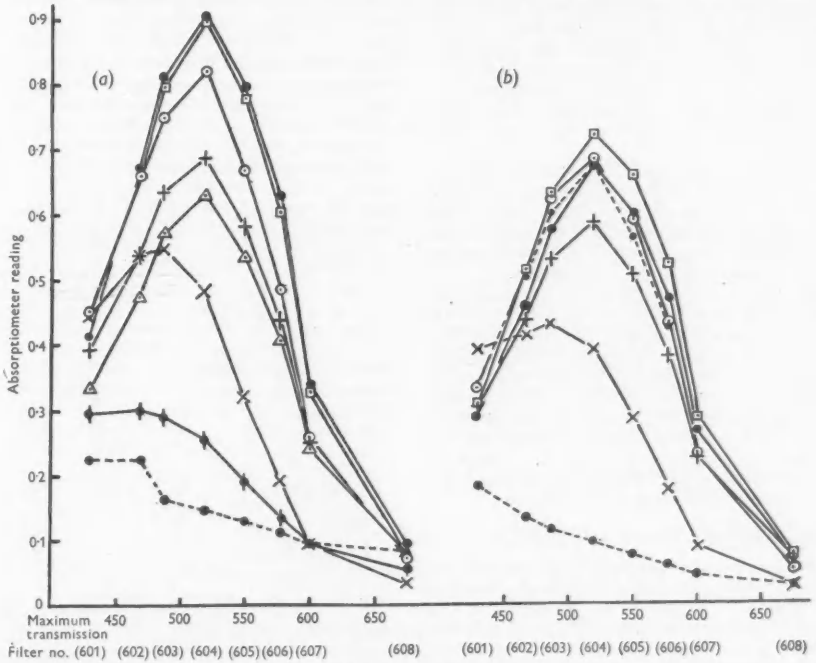


Fig. 8. Light absorption curves of pigments formed by enzymic oxidation of (*a*) catechol and (*b*) homocatechol in the presence of nitrogenous bases. Ordinates, absorptiometer readings; abscissae, wave lengths of maximum transmission of the filters used, the serial number of each filter (Ilford spectrum series) being given in the appropriate position under the wave-length scale. The bases used were ●—●, proline; ⊡—⊡, hydroxyproline; ⊙—⊙, hydroxyproline ethyl ester; +—+, pyrrolidine; ×—×, glycine; △—△, dimethylamine; ⧫—⧫, methylamine. The broken line at the foot of each set of curves indicates the readings in a control in which no base had been present. The measurements were made at a dilution corresponding to 0·0002 M in terms of the amount of *o*-diphenol used, and in a 1 cm. cell. The upper broken line in (*b*) indicates the readings given by a 0·0002 M solution of the synthetic quinone I.

colour intensity (in terms of actual absorptiometer reading) is plotted against the ratio mol. base/mol. *o*-diphenol of the reaction mixture. It will be noted that with catechol as substrate increasing the concentration of nitrogenous base above an equimolar proportion results in little increase in pigment formation in the case of proline, hydroxyproline and hydroxyproline ethyl ester, and even with homocatechol more than 50 % of the maximum colour is produced by these substances at a 1:1 ratio. This is a strong indication that the formation of the pigment requires only 1 mol. of nitrogenous base/mol. of *o*-diphenol. It seems likely that a condensation reaction takes place between 1 mol. of nitrogenous substance, through its imino group, and 1 mol. of the primary oxidation product, *o*-benzoquinone (Pugh & Raper, 1927), to give the leuco form of the pigment, which is then changed into the coloured form by the utilization of 1 atom of oxygen. In these experiments hydroxyproline ethyl ester behaved uniquely in that increasing its relative concentration above the 1:1 ratio led to a diminution in the amount of pigment found. This effect was much more striking with catechol than with homocatechol as the substrate, and was negligible at pH 6 as the appropriate curve in Fig. 7 indicates. It appears to be due to an instability of the pigment in the presence of excess hydroxyproline ethyl ester. The curves of Fig. 7 do not give such a definite indication as to the molecular proportions involved in the reactions with pyrrolidine, glycine and the methylamines. Higher concentrations of these bases were necessary for effective reaction, and it is uncertain whether an approach to quantitative conversion of *o*-diphenol to pigment was achieved even at the highest concentrations used.

The colours given by catechol and homocatechol with the same base were not distinguishable in tint to the naked eye. The various substances containing a secondary amino group appeared to give an identical colour in the reaction, whilst those with a primary amino group gave quite a different hue. To record this more objectively, absorptiometer readings were taken, using in turn each filter of the Ilford spectrum series, and the results are shown in Fig. 8. The pigments derived from secondary amines all give curves of a very similar form with maximum absorption when the green (no. 604) filter was used. The glycine-pigment curves are clearly different and show maximal absorption with the blue-green filter (no. 603), and methylamine resembled glycine in its behaviour in this respect. These facts suggest that the pigments derived from the various secondary amines are analogous in structure, but of course do not necessarily preclude a similar structure in the glycine pigment.

Liberation of hydrogen ion during pigment formation

The method used for this study has already been described. The experiments were carried out at room temperature, about 20°. The addition of enzyme to the aerated solution of *o*-diphenol and base caused a rapid liberation of hydrogen ion corresponding in time with the rapid phase of O_2 absorption and ceasing abruptly when pigment formation was completed. The course of hydrogen-ion liberation in one such experiment is shown in Fig. 9, and the relationship between total hydrogen ion liberated and amount of *o*-diphenol oxidized may be seen in the summarized data of Table 2. It is interesting to note that there was a significant liberation of hydrogen ion during the oxidation even in the absence of any nitrogenous base, but the source of this hydrogen ion is at present unknown. The data given in Table 2 are in accordance with the view that when a base was present the amine group was involved in the pigment-forming reaction, and was converted into a less basic form unionized at pH 7, so that an equivalent of hydrogen ion was liberated. Direct quantitative relation of the measured hydrogen-ion liberation to the

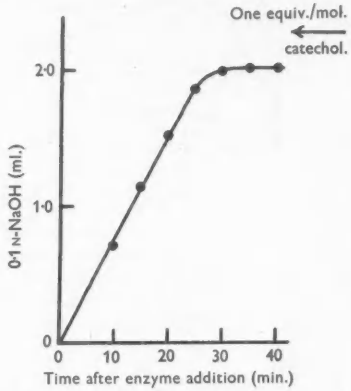


Fig. 9. Hydrogen-ion liberation during the oxidation of 0·227 mmol. catechol by tyrosinase in the presence of 0·227 mmol. hydroxyproline. The reaction took place in unbuffered solution at pH 7·0, and the ordinates represent the addition of standard alkali necessary to maintain the pH constant during the reaction.

Table 2. *Liberation of hydrogen ion during the oxidation of catechol and homocatechol by tyrosinase in the presence of nitrogenous bases*

	H^+-ions liberated/mol. of			
	Catechol		Homocatechol	
Molar ratio base/phenol	1:1	10:1	1:1	10:1
Nitrogenous base:				
Proline	0·76	0·96	0·48	0·81
Hydroxyproline	0·91	1·02	0·72	0·90
Hydroxyproline ethyl ester	0·89	0·95	0·79	0·83
Pyrrolidine	0·55	0·82	0·47	0·73
Dimethylamine	—	0·79	—	—
Glycine	0·52	0·79	—	0·68
Methylamine	—	0·53	—	—
None	0·18		0·23	

amount of amino group reacting depends upon the assumption that the reacting group is fully ionized in its initial, and completely unionized in its final, stage. Of the bases used, only hydroxyproline ethyl ester (pK = *c.* 7·5) has its basic group appreciably unionized at pH 7, and in this case only, therefore, the experiments were carried out at pH 6. In the cases where pigment formation was most efficient, the hydrogen-ion liberation approached 1 equiv./mol. of *o*-diphenol initially present when the base/*o*-diphenol ratio was 10:1, but did not exceed this even when 90 % of the maximum was achieved at a 1:1 ratio. It is clear then that only 1 mol. of base/mol. of *o*-diphenol participates in the reaction. The

smaller yields of hydrogen ion in the case of pyrrolidine, glycine and methylamines correspond with the lesser degree of reactivity of these compounds in pigment formation already pointed out in connexion with the colour production curves of Fig. 7.

Properties of the pigments

Since the preliminary report on the formation of the proline and hydroxyproline pigments was made (Jackson & Kendal, 1940) many attempts at isolation of the pigments have been carried out without success. They are very soluble in water, methanol and ethanol, and are not extracted from aqueous solution into any of the common solvents. They are moderately stable within a narrow range of pH between 6 and 7, but even at pH 7 about 45% of the colour intensity disappears from the solution in 48 hr. at room temperature. Outside this range the pigments are very unstable, and this is undoubtedly the main difficulty in attempts at isolation.

The addition of strong acid to a solution of the pigment formed from catechol and imino acid causes rapid and irreversible decolorization, and the kinetics of this decomposition have been studied. Catechol (1 mg.) was oxidized under the conditions already described, with excess of enzyme and excess of the imino acid present. The pigment-containing reaction mixture (4 ml.) was then diluted with 0·1 M-phosphate buffer of pH 7 to a volume of 50 ml. Samples of this solution (5 ml.) were mixed with water and varying amounts of 0·1 N-HCl, to give in each case a final volume of 15 ml. Immediately after the addition of the acid the mixture was placed in the absorptiometer cell and readings were taken at timed intervals. When the desired number of observations had been made the pH of the solution was determined with the glass electrode. At pH's of less than 3·0 the pigment was found to be completely decolorized within 10 min. The results are summarized in Fig. 10, where the logarithm of the absorptiometer reading is plotted against the time which had elapsed since the pH of the pigment solution was changed from its original value of 7·0 to the value specified alongside each curve. Since the logarithmic plots are straight lines, the conversion of the pigment into colourless products has the appearance of being a monomolecular reaction. However, the relative rates of destruction at different pH's indicate that the disappearance of the colour follows the course of a bimolecular reaction in which $dx/dt = k[H^+](a-x)$, where a is the amount of pigment originally present (as measured by absorptiometer reading) and x is the amount converted to colourless products in time t. The values of k in this expression, calculated from the data of Fig. 10, are given in Table 3. The acid decomposition of the pigment formed, when catechol was oxidized in the presence of one of the partially purified fractions of mushroom extract, was examined in the same way. Log k was found to be 2·75, which is in agreement with the belief that the effective substance in the fraction was proline. In similar experiments with the pigments from homocatechol and hydroxyproline or hydroxyproline ethyl ester values for log k of approx. 3·1 and 0·4 were respectively obtained. The decolorization of the homocatechol-hydroxyproline pigment, therefore, proceeds at a rate comparable with that of the corresponding catechol pigment, whilst in the case of the ester pigment, the rate is of a different order of magnitude, some hundreds of times slower. This suggests that in the latter case a different mechanism is involved; it may be that the overall rate is determined by the rate of hydrolysis of the esterified carboxyl group present in this pigment. The pigment formed with pyrrolidine gave quite different results. Acidification of a sufficient degree produced, in this case, an instantaneous change from purple to yellow, and at no pH between 1·0 and 7·0 was any further change in the absorptiometer reading detectable in a period of 10 min. The colour at pH 1·0 was a clear yellow with no trace of pink, and intermediate tints were given as the pH was raised until the full purple colour appeared when a pH of 3·0 was exceeded. The change was reversible and the pigment behaved as a typical indicator. A rough calculation, from the absorptiometer readings, of the proportions of the two forms present at different pH's suggested that the colour change was associated with the ionization of a group with a dissociation exponent of about 2·1, whether the pigment had been prepared from catechol or from homocatechol.

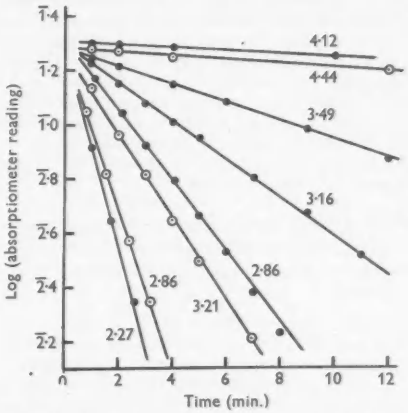


Fig. 10. Decolorization of pigments in acid solution. The logarithm of the absorptiometer reading is plotted against the time which had elapsed since the pH of the pigment solution was changed from its initial value of 7·0 to the value specified alongside each curve. The pigments were formed from ●—●, catechol + hydroxyproline; and ⊙—⊙, catechol + proline.

Table 3. *Velocity constants of the pigment decolorization reaction*

Proline pigment		Hydroxyproline pigment	
pH	log k	pH	log k
2·86	2·69	2·27	2·19
3·21	2·76	2·86	2·33
4·44	2·67	3·16	2·36
—	—	3·49	2·39
—	—	4·12	2·18
Mean	2·71	Mean	2·29

The decomposition of the proline and hydroxyproline pigments in acid solution is accompanied by the evolution of CO_2. This was demonstrated in experiments in the Warburg apparatus. O_2 uptake during pigment formation was determined in the manner already described. A second manometer with the same reaction mixture had no KOH in its inset cup, and within the vessel was a dangler containing 0·2 ml. of

2N-HCl, which was tipped into the reaction mixture after the O_2 uptake accompanying pigment formation was complete. From the resultant increase in pressure the volume of CO_2 liberated was calculated in the usual way, due allowance being made for the small initial CO_2 content of the reaction mixture which was determined in blank experiments. A few similar experiments were carried out in which pyrrolidine, glycine or alanine was used instead of the imino acid. In some of these the NH_3 content of the reaction mixture at the end of the acid treatment was determined, the reaction mixture being made alkaline, the NH_3 drawn over in an air stream into standard acid and estimated by back titration. The results of a number of such experiments are given in Table 4. It is evident from the table that the CO_2 liberation on acidification of the imino acid pigments approaches 1 mol./mol. of catechol used for pigment formation. Since this is not observed in the case of the pyrrolidine pigment (where the small figure obtained is not with certainty outside the limits of experimental error) it is probable that the CO_2 liberation is the result of instability of the carboxyl group in the pigment in acid solution. In the experiments with the glycine and alanine pigments CO_2 liberation on acidification lagged markedly behind O_2 uptake at the moment of acidification. This may be merely a reflexion of the failure of the approach to quantitative formation of pigment from catechol in the case of these amino-acids.

Table 4. *Oxygen uptake during pigment production, CO_2 formation on acidification of pigment solutions, and ammonia content of reaction mixtures after this treatment*

(Pigment was formed from 4·55 μmol. catechol in each case. In Exps. 1–4, 22·7 μmol. nitrogenous base were present, and the reaction mixture was acidified after O_2 absorption had ceased. In Exps. 5–8, 91 μmol. nitrogenous base were present, and the reaction mixtures were acidified at the times indicated. Zero time was the moment of addition of enzyme. The figures given are mol./mol. of catechol.)

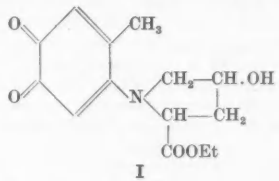
Exp. no.	Nitrogenous base	Time (min.)	O_2	CO_2	NH_3
1	Proline	—	0·99	0·92	—
2	Hydroxyproline	—	1·01	0·75	—
3	Mushroom fraction	—	1·01	0·92	—
4	Pyrrolidine	—	1·01	0·09	—
5	Glycine	17	1·10	0·20	0·61
	,,	211	3·23	0·97	2·99
6	Glycine	10	0·99	0·15	0·20
	,,	70	2·12	0·53	1·85
	,,	200	3·23	0·83	3·25
7	Alanine	10	0·88	0·64	0·00
	,,	140	1·06	0·77	0·26
	,,	220	1·21	0·83	0·51
8	Proline	210	1·01	0·95	0·00

The amounts of NH_3 liberated from the reaction mixtures were in most cases so small as to be within the limits of experimental error of estimation. Only in the later stages of the reaction with glycine can NH_3 liberation be claimed to have been with certainty demonstrated to occur on a scale too great to be accounted for as possibly the result of destruction of the pigment itself by the acid or alkali treatment. It is concluded that this NH_3 was set free in the reaction prior to the acidification; the amount of it appears to have been about 1 mol./mol. of oxygen absorbed. The probable nature of the reactions involved will be referred to in the discussion.

SYNTHESIS OF PIGMENT

In view of the difficulties met with in attempts to isolate the pigments, believed to be due to the instability of the pigments in aqueous solution, some attention was given to the possibility of preparing such pigments by ordinary chemical methods in non-aqueous media. A crystalline pigment was obtained from homocatechol and hydroxyproline ethyl ester in the following way.

Homocatechol (0·31 g.) and hydroxyproline ethyl ester hydrochloride (0·45 g.) were dissolved in dry ethanol (25 ml.) with gentle warming to facilitate solution of the hydrochloride. The addition of dried Na_2SO_4 (3·5 g.) followed by dry, alkali-free Ag_2O (1·5 g.) resulted in the development of the expected purple-red colour. The mixture was shaken for 10–15 min. Ag_2O, Ag and Na_2SO_4 were removed by filtration and the filtrate cooled in ether-solid CO_2. Scratching promoted the separation of minute rosettes of needles. After cooling (1 hr.) the crystals were filtered off and washed with light petroleum (b.p. 40–60°) until the washings were colourless, and then dried *in vacuo*. Recrystallization from absolute ethanol-ether gave 130 mg. of purplish-black glistening crystals, m.p. 125–126°. Found: C, 60·2; H, 6·3; N, 5·0. $C_{14}H_{17}O_5N$ requires C, 59·8; H, 6·4; N, 5·0%. The product is therefore taken to be 4-(*4′-hydroxy-2′-carbethoxy-1′-pyrrolidyl*)-5-*methyl*-o-*benzoquinone* with the structure I. It will be referred to in this paper as 'the quinone I'.



I

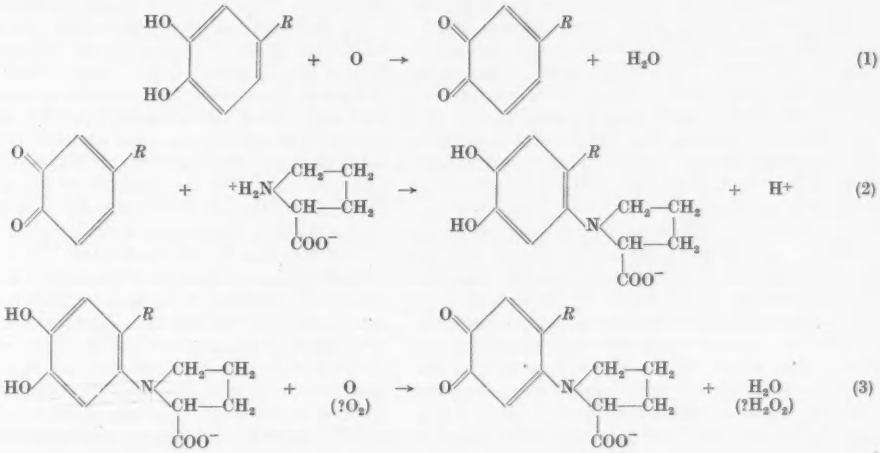
Using catechol instead of homocatechol, or free hydroxyproline or proline instead of the ethyl ester, pigments are readily formed under these conditions, but it has not so far proved possible to isolate them in a pure state. It appears that the presence in the pigment molecule of either the free carboxyl or an unsubstituted position para to the quinonoid oxygen confers upon it an extra degree of instability which renders its isolation more difficult.

Aqueous solutions of the crystalline pigment resemble very closely in colour those of the enzymically produced imino acid pigments, as may be seen on comparing the appropriate curves in Fig. 8. They are all decolorized by ascorbic acid, and in each case the leuco form of the pigment is spontaneously re-oxidized on shaking with air (e.g. after removal of enzyme by adsorption on alumina). A striking property of the imino acid pigments formed in the

enzyme reaction is that they are able to effect a non-enzymic oxidation of glycine by atmospheric oxygen, and this property is also shared by the quinone I. These facts strongly suggest that all these pigments have essentially the same structure.

DISCUSSION

The quantitative observations which have been made on oxygen uptake and hydrogen-ion liberation accompanying pigment formation, on the relationship between intensity of colour developed and imino acid concentration, and on CO_2 liberation during acid decomposition of the pigment, suggest that the sequence of reactions involved may be represented as follows:



When $R = H$ (catechol) the sequence is the same as when $R = CH_3$ (homocatechol), although the speed of the actual pigment-forming reaction (2) is greater. Of these reactions (1) is now generally accepted as the first step in the oxidation of catechol by tyrosinase. In the absence of other substances, the *o*-quinone formed takes part in further reactions which are associated with the uptake of about 1 atom of oxygen per mol. In the belief that this further uptake is precisely 1 atom/mol., Wagreich & Nelson (1938) have suggested that the product is a hydroxy-*o*-quinone which then polymerizes to give the brownish-black end product. We have frequently observed a total O_2 absorption of up to 2·3 atoms/mol. catechol, and it seems likely that the end stages are more complicated and that they may involve, perhaps in addition to hydroxyquinone formation, some degree of condensation of the type shown by Westerfield & Lowe (1942) to occur during the oxidation of *p*-cresol by peroxidase. Evidence for the accumulation of *o*-quinone during the early stages of the oxidation of catechol by tyrosinase has been provided by Dawson & Nelson (1938), and the reactions which bring about the disappearance of *o*-quinone are relatively slow compared with primary oxidation of catechol or homocatechol.

In the presence of primary or secondary amines the effectiveness of pigment formation must depend in the first place on the relative speeds of the reaction of *o*-quinone with amine, and the reactions by which the *o*-quinone is removed in the absence of amine. The condensation (reaction 2) under the conditions of our experiments appears to have been sufficiently rapid in the cases of proline, hydroxyproline, and hydroxyproline ethyl ester for pigment formation from catechol to have exceeded 90 %, from homocatechol 80 %, of the theoretical requirement according to the reaction scheme proposed. This conclusion is based on the hydrogen-ion liberation during the reactions. The reaction with pyrrolidine, glycine and the methyl amines was much slower and a greater fraction of the *o*-diphenol present was presumably converted into the same end products as are obtained in the absence of nitrogenous base.

It is well established that when catechol is oxidized by tyrosinase in the presence of aniline, dianilinoquinone is formed almost quantitatively (Pugh & Raper, 1927). It is not clear why in the case of the imino acids, reaction (2), analogous to the first stage of dianilinoquinone formation, having taken place, the quinone derived from the product by reaction (3) does not then condense with a second molecule of imino acid. No study of the products of the reaction between quinone and free aliphatic

amino-acids appears to have been made. Fischer & Schrader (1910), allowing glycine ethyl ester to react with *p*-benzoquinone in ethanol, isolated the product and showed it to be the diethyl ester of diglycinoquinone. These workers prepared in the same way the ester of dialaninoquinone, and from *p*-toluquinone the ester of diglycinotoluquinone. The formation of the *di*glycino compound from toluquinone in this case is interesting, particularly in view of Suchanek's (1914) finding that whereas *p*-benzoquinone with *p*-aminobenzoic acid gave a mixture of mono- and di-anilinoquinones, from toluquinone under the same conditions only the monoanilinoquinone was formed, while xyloquinone gave no anilinoquinone. Studies of this kind do not seem to have been made with *o*-benzoquinone, but from a reaction mixture in which *p*-cresol had been oxidized by tyrosinase in the presence of aniline Pugh & Raper (1927) isolated a dianilinoquinoneanil. The conditions under which condensations of the anilinoquinone and quinone-anil types take place do not yet appear to be very well defined. But as regards the former type of condensation the available evidence suggests that it takes place with a strong preference in a position para to a quinonoid oxygen in an *o*-quinone, and ortho to a quinonoid oxygen in *p*-quinone, without ruling out the possibility of a further condensation in a position not so situated. In the formation of the crystalline pigment prepared synthetically from homocatechol and hydroxyproline ethyl ester in the present series of experiments, the condensation was undoubtedly limited to 1 mol. of base per mol. homocatechol. There is no reason to question the identity of this pigment with the pigment formed from the same precursors enzymically and both resemble so closely the pigments formed from catechol and the secondary amines in both properties and manner of formation, that it is certain that they all have the same general structure. In the case where catechol is the substrate, it seems possible that steric hindrance by the proline residue may be responsible for the prevention of the condensation of a second molecule of imino acid in the unoccupied position para to quinonoid oxygen. The reaction of *o*-quinone with glycine appears to take place less rapidly than the corresponding reaction with imino acid, and the observations on pigment formation in the catechol-tyrosinase-glycine system do not indicate clearly the molar ratio of catechol and glycine reacting to form the pigment. It is likely that the chief reaction of glycine with *o*-quinone in aqueous solution is the same as in the case of the imino acids so that a monoglycinoquinone is formed.

A suggestion that the enzyme tyrosinase was able to bring about the oxidative deamination of amino-acids was put forward by Chodat & Schweizer (1913) and received some support from other workers. Happold & Raper (1925), however, made it clear that well dialyzed tyrosinase preparations had no effect on amino-acids other than tyrosine itself, but demonstrated liberation of ammonia from glycine and alanine by tyrosinase plus *p*-cresol, catechol or phenol. They attributed the oxidation of amino-acid to a secondary reaction of this with *o*-quinone formed by the enzyme, and showed that *o*-quinone reacted non-enzymically with amino-acids and liberated ammonia. The fact that the reaction mixtures were 'deeply pigmented' was mentioned, but without comment. The observations recorded in this paper now indicate that *o*-quinone condenses with 1 mol. of amino- or imino acid to give a leuco pigment which is readily autoxidizable in air. Whether water or hydrogen peroxide is the other end product of this autoxidation has not yet been determined. In the case of the imino acid pigments no further reaction takes place even if an excess of the imino acid is present. When the pigment is formed from glycine, and to a lesser extent when it is formed from other amino-acids, excess amino-acid is oxidized with liberation of ammonia. The relation between oxygen used and ammonia liberated in experiments of this kind with glycine (Table 4) are such as would be expected if the main end products of the oxidation of glycine were ammonia and oxalic acid. Experiments now in progress, which will be described in detail in another paper, indicate that the imino acid pigment reacts with and brings about the oxidation of glycine, and that no enzyme is required for this reaction. It therefore appears probable that the effective oxidant of excess amino-acid is a pigment with a structure corresponding to that of the synthetic quinone I. This may react with further amino-acid, dehydrogenating it to give imino acid which then decomposes with liberation of ammonia. The leuco pigment simultaneously formed is spontaneously re-oxidized by oxygen. But the possibility that an intermediate stage in the dehydrogenation is a condensation of a second molecule of amino-acid into the pigment molecule should be borne in mind.

An interesting problem arises in connexion with the decomposition of the proline and hydroxyproline pigments in acid solution. This decomposition has been found to be irreversible and to be accompanied by the loss of 1 mol. of carbon dioxide. Simple decarboxylation of the proline pigment should, however, yield the corresponding pyrrolidine pigment. However, the latter when prepared in solution directly from catechol and pyrrolidine was found to be reasonably stable, though yellow in colour, in acid solution, giving the purple form reversibly on raising the pH, and hence was clearly not identical with the decomposition product of the proline pigment. It is not at present possible to offer an adequate solution of this problem.

SUMMARY

1. The component of crude mushroom extracts which is responsible for the appearance of a purple colour when such extracts oxidize catechol has been identified by isolation of L-proline.

2. The pigment-forming reaction has been studied in respect of colour intensity developed, oxygen absorbed and hydrogen ion liberated, in systems containing purified tyrosinase, catechol or homocatechol, and one of the following: proline, hydroxyproline, hydroxyproline ethyl ester, pyrrolidine, glycine, dimethylamine and methylamine. A few experiments were also carried out with alanine, glutamic acid and arginine. The same intense purple colour was obtained in each case in which the nitrogenous substance present had a secondary amino group; the compounds with primary amino nitrogen gave a much less intense orange-red colour.

3. From homocatechol and hydroxyproline ethyl ester, by oxidation with silver oxide in ethanol, 4-(4′-hydroxy-2′-carbethoxy-1′-pyrrolidyl)-5-methyl-*o*-benzoquinone has been prepared in a pure state. It appears to be identical with the pigment formed from the same precursors in the enzymic reaction mixture, and it is concluded that all the pigments have an analogous structure, and that the pigment-forming reaction is essentially a condensation between 1 mol. of *o*-quinone and 1 mol. of nitrogenous base to give a leuco pigment which is spontaneously oxidized to the coloured form by oxygen.

4. In the case of glycine, when this is present in excess, the formation of pigment is followed by oxidation of the excess amino-acid, with liberation of ammonia. Further oxidation of this type does not take place in the case of the imino acid pigments.

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β-Glucuronidase as an Index of Growth in the Uterus and other Organs

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The β-glucuronidase activity of mouse liver or kidney has been shown to be related to the degree of cell proliferation in progress (Levvy, Kerr & Campbell, 1948). It was suggested that the rise in uterine glucuronidase observed after administration of oestrogens to ovariectomized mice (Fishman & Fishman, 1944; Fishman, 1947) could also be explained by cell proliferation.

A comparative study has been made of the kinetics of hydrolysis of phenylglucuronide by β-glucuronidase from mouse uterus, liver and kidney, and of the effects on the enzyme activities of various measures designed to produce proliferative changes in one or more of these organs. Mills (1947) showed that ox-spleen glucuronidase could be separated into two fractions, *A* and *B*, with slightly different pH optima for the hydrolysis of menthylglucuronide. Both these fractions have been found in mouse liver and kidney, while uterine glucuronidase appears to be composed entirely of *A*. No evidence has, however, been obtained to suggest that the effect of an extrinsic agent on the glucuronidase activity of an organ is dependent upon which fraction happens to be present. As in liver and kidney, changes in the enzyme level in uterus resulting from a variety of causes appear to be associated with alterations in growth.

In the course of these experiments, some unexpected changes in glucuronidase activity were encountered. In ovariectomized mice, measures

designed to cause a rise in glucuronidase in liver also produced an increase in uterus, whilst an elevated enzyme activity in liver as well as in uterus was seen after administration of oestrone. These effects have been further investigated.

A preliminary account of part of this work has been published elsewhere (Kerr & Levvy, 1948).

EXPERIMENTAL

Enzyme assay. To permit determination of β-glucuronidase activity in a single mouse uterus, the procedure previously described (Kerr, Graham & Levvy, 1948) was adapted for use with the microcells of the Spekker absorptiometer. As before, the tissue homogenate was freed from inactive protein by maintaining it at pH 5·2 and 38° for 30 min., and the enzyme was precipitated by addition of an equal volume of saturated $(NH_4)_2SO_4$ solution. The enzyme was dissolved in a volume of water such that 0·2 ml. of the resulting solution gave a final reading of 2–4 μg. phenol after correction for blanks. This volume of the enzyme solution was added to 0·1 ml. 0·06 M-phenylglucuronide and 0·1 ml. 0·1 M-citrate buffer at the appropriate pH (see below). After incubation of the hydrolysis mixture for 1 hr. at 38°, 0·5 ml. of a 1 in 5 dilution of Folin-Ciocalteu reagent was added. Protein was removed by centrifuging, and 0·5 ml. of supernatant transferred to a tube containing 0·5 ml. 1·33 N-Na_2CO_3. Colour development was carried out for 20 min. at 38°, and the results were read from a graph constructed with standard phenol solutions put through the same procedure. Assays were done in duplicate, and enzyme and substrate controls were performed as usual. This technique was also adopted for determinations of liver and kidney glucuronidase in the experiments described below, and results are shown in terms of glucuronidase units (G.U.)/g. moist tissue, where 1 G.U. liberates 1 μg. phenol under the standard conditions.

Weight of uterus. Before determining the moist weight of uterus, the tissue was freed from intrauterine fluid by pressing it between pieces of filter paper. The figure then obtained was found to bear a constant relation to the weight after drying at 110° for all conditions of the uterus. No error was introduced into the enzyme assay since the intrauterine fluid contained no detectable amounts of glucuronidase.

RESULTS

Kinetic studies. The pH-activity curve for hydrolysis of phenylglucuronide by mouse-kidney glucuronidase resembled those previously obtained for liver and spleen (Kerr *et al.* 1948) in having two peaks, one at pH 4·5 and the other at pH 5·2. In the case of uterus, however, the activity curve was symmetrical about pH 4·5, and this was still true when the initial purification of the homogenate was omitted. Changes in the enzyme activity in liver, kidney or uterus were not associated with any alteration in the shape of the pH-activity curve (Figs. 1–3). To cover the pH range it was necessary in the case of uterus to pool preparations from two or more mice. For liver and kidney this was only necessary with infant mice. The high figure for uterine glucuronidase in infant mice supports the view that in this organ, as in others, the activity of the enzyme is a measure of growth processes.

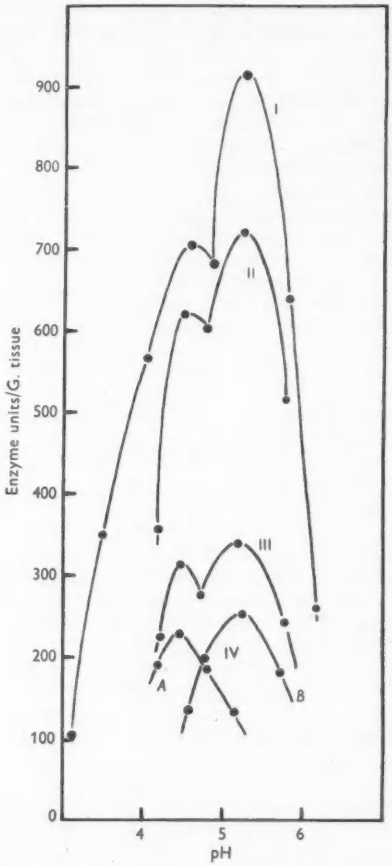

Fig. 1. pH-Activity curves for liver glucuronidase. I, 6-day-old mice; II, adult, 1 day after subcutaneous injection of 5 g. CCl_4/kg.; III, normal adult; IV, the same preparation as III after separation of fractions *A* and *B*.

It was considered that the shapes of the curves for the hydrolysis of phenylglucuronide by mouse liver or kidney indicated the presence of the two glucuronidase fractions found by Mills (1947) in ox spleen, and his technique was applied to their separation. After the preliminary removal of inactive protein by incubation for 30 min. at pH 5·2, the homogenate was made 31·5% saturated with ammonium sulphate. The precipitate thus obtained was devoid of glucuronidase activity. On bringing the preparation to 38·5% saturation with ammonium sulphate, a

large part of the enzyme was precipitated (fraction *A*), whilst all residual activity was removed from solution when the ammonium sulphate concentration was increased to 44·0% saturation (fraction *B*). The separation of the two peaks in the pH-activity curves for liver and kidney achieved in this way is illustrated in Figs. 1 and 2, and it appears that the shapes of the original curves can in fact be explained in terms of Mills's (1947) two fractions. In the fractionation of uterine preparations, all enzyme activity was found in fraction *A*.

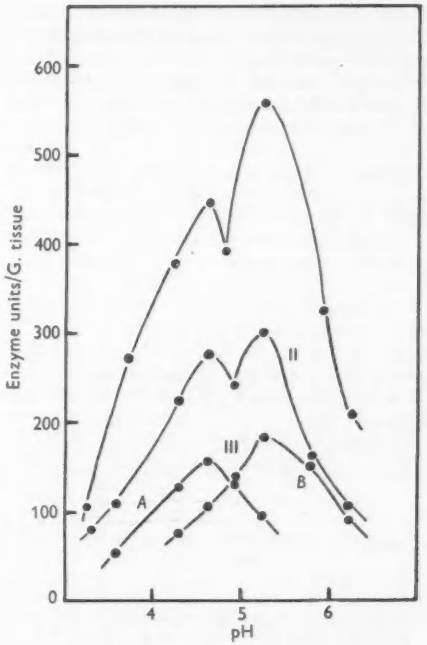

Fig. 2. pH-Activity curves for kidney glucuronidase. I, 6-day-old mice; II, normal adult; III, the same preparation as II after separation of fractions *A* and *B*.

The effect of varying the substrate concentration was studied with uterine enzyme and with fractions *A* and *B* from liver. In every case the activity curve closely resembled that obtained with liver before separation of the two fractions (Kerr *et al.* 1948). K_m, the substrate concentration at which half the observed maximum velocity of hydrolysis was attained, was approximately the same for the two glucuronidase fractions at the figure for the total enzyme in liver (0·0035 M).

Mills (1948) has recently published figures for the pH optima in the hydrolysis of phenylglucuronide by his two glucuronidase fractions from ox spleen, and these correspond exactly with the peaks in the pH-activity curves for mouse liver, kidney and spleen.

The effects of various agents on fractions A and B in liver and kidney

The possibility was considered that differences between glucuronidase fractions *A* and *B* in their distribution and response to extrinsic agents might explain the selective actions of such agents on various organs. The nature of the effect of carbon

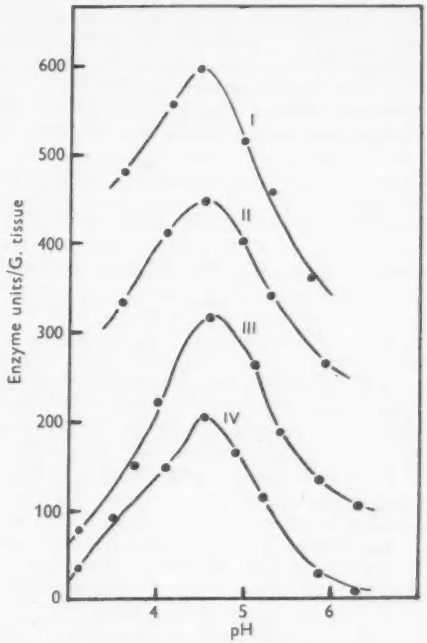

Fig. 3. pH-Activity curves for uterine glucuronidase. I, 10-day-old mice; II, ovariectomized adults, 3 days after subcutaneous injection of 1·7 mg. oestrone/kg.; III, normal adults; IV, ovariectomized adults.

tetrachloride on the pH-activity curve for liver (Fig. 1) renders this possibility unlikely, since both fractions were equally affected in the increase in activity. Carbon tetrachloride is known to be without effect on kidney glucuronidase (Levvy *et al.* 1948), although this organ resembles liver in the composition of the enzyme. In spite of these findings a great many more experiments were done before the possibility just outlined was rejected. In these experiments, the homogenate from each organ was divided into two portions, in one of which *A* and *B* were separated as described above and determined at their respective pH optima, 4·5 and 5·2. The other

portion of the homogenate was brought to 50% saturation with ammonium sulphate and the total enzyme thus precipitated was determined at pH 5·2. Average results for liver and kidney under a variety of conditions are shown in Table 1. Since *A* was determined at a different pH from the total activity, figures for the latter do not agree with the sums of the two fractions. One point not brought out in the table is that individual mice, treated and untreated, showed considerable variation in both liver and kidney in the ratio of the two fractions. Very occasionally, one animal in a group displayed complete lack of *A* or *B* in one of the two organs examined, unaccompanied by any compensatory increase in the activity of the remaining fraction. When this occurred, the size of the group was reduced by one in calculating the average and standard error for the fraction in question, as shown in Table 1.

cutaneous injection of carbon tetrachloride in olive oil were in entire agreement with the conclusions arrived at above, in that this agent showed no discrimination between the two fractions in liver, and was without effect on either in kidney. After subcutaneous injection of mercuric nitrate as an aqueous solution, *A* and *B* rose and fell together in kidney as repair processes became active and were completed. Since mercuric nitrate has little effect on liver (see Table 3), its action on this organ was not studied in the present experiments.

Changes in liver glucuronidase after subcutaneous injection of chloroform in olive oil closely resembled, as one might expect from previous work, those produced by carbon tetrachloride. Taking the results as a whole, there was a suggestion that fraction *A* returned to normal more rapidly than *B*. Before dealing with the effects of chloroform on kidney

Table 1. *Effects of various agents on glucuronidase fractions A and B in liver and kidney*

(All values are given as mean ±S.E., followed (in parentheses) by the number of animals in the group.)

			G.U./g. moist tissue								
			Liver			Kidney		
Agent	Sex*	Days after treatment	*A*†	*B*‡	Total‡	*A*†	*B*‡	Total‡
None	M.	—	106±17 (6)	223±12 (6)	281±20 (6)	124±16 (6)	123±38 (6)	266±31 (6)
	F.	—	115±12 (6)	247±38 (6)	334±48 (6)	109±8 (6)	118±16 (6)	266±39 (6)
	cM.	—	127±13 (6)	217±18 (6)	301±18 (6)	130±22 (6)	194±18 (6)	286±20 (6)
	cF.	—	116±12 (6)	167±47 (6)	250±49 (6)	113±10 (6)	130±22 (6)	261±43 (6)
Carbon tetrachloride (5·3 g./kg.)	M.	1	545±18 (3)	654±40 (2)§	830±62 (3)	122±27 (3)	150±61 (3)	206±28 (3)
	M.	4	364±58 (3)	537±27 (3)	763±65 (3)	118±19 (3)	138±18 (3)	257±19 (3)
	cF.	4	499±109 (3)	588±24 (3)	664±57 (3)	126±14 (3)	158±6 (3)	235±23 (3)
	cF.	7	517±27 (3)	568±35 (3)	693±31 (3)	127±14 (3)	186±70 (3)	270±42 (3)
Mercuric nitrate (20 mg./kg.)	M.	3	—	—	—	269±35 (3)	319±55 (3)	463±59 (3)
	M.	6	—	—	—	122±14 (3)	158±15 (2)§	277±19 (3)
	F.	3	—	—	—	224±24 (3)	351±45 (3)	491±62 (3)
	F.	6	—	—	—	122±10 (3)	124±26 (3)	237±61 (3)
Chloroform (2 g./kg.)	M.	1	343±188 (6)	766±147 (6)	881±163 (6)	134±19 (6)	121±17 (6)	206±19 (6)
	M.	4	260±42 (3)	561±44 (3)	766±46 (3)	128±18 (3)	234±22 (3)	302±12 (3)
	M.	7	184±29 (6)	559±38 (6)	650±72 (6)	132±29 (6)	395±54 (6)	524±38 (6)
	F.	1	537±64 (6)	410±194 (6)	812±91 (6)	173±10 (6)	135±9 (6)	227±23 (6)
	F.	4	271±45 (3)	570±52 (3)	696±61 (3)	126±19 (3)	148±35 (3)	238±27 (3)
	F.	7	151±42 (6)	559±106 (6)	705±64 (6)	108±16 (6)	223±78 (6)	310±55 (6)
	cM.	1	453±39 (3)	537±25 (3)	659±174 (3)	119±12 (3)	141±17 (3)	221±38 (3)
	cM.	7	152±20 (3)	549±42 (3)	673±32 (3)	132±20 (3)	124±22 (3)	238±30 (3)
	cF.	1	277±22 (2)§	594±32 (2)§	515±23 (3)	187±21 (6)	256±29 (6)	398±67 (6)
	cF.	4	224±25 (3)	510±20 (3)	620±26 (3)	189±15 (3)	247±18 (3)	352±24 (3)
	cF.	7	359±130 (5)	569±39 (5)	657±78 (5)	175±32 (7)	260±69 (7)	359±67 (7)

* c = castrated.
† One G.U. (glucuronidase unit) liberates 1 μg. phenol from 0·015 M-phenylglucuronide in 1 hr. at 38° and pH 4·5.
‡ One G.U. (glucuronidase unit) liberates 1 μg. phenol from 0·015 M-phenylglucuronide in 1 hr. at 38° and pH 5·2.
§ One animal in group devoid of this fraction.

From Table 1, it appears that in either liver or kidney the distribution of glucuronidase activity between the two fractions was on the average the same for normal male and female mice. Castration 3–4 weeks before sacrifice had no marked effect on the results in either sex.

Results obtained during the prolonged rise in liver glucuronidase activity which follows subglucuronidase, it is necessary to consider the influence of sex on the susceptibility of the mouse kidney to chloroform necrosis. Eschenbrenner (1944) found that chloroform caused renal necrosis in male, but not in female mice, and it was shown later (Eschenbrenner & Miller, 1945) that the effect in males was prevented by castration at an early age. The sex-linked nature of this response was associated with

differences in the structure of Bowman's capsule described by Crabtree (1941), but chloroform necrosis, which involved the convoluted tubules, did not extend to the capsule itself. In previous work on glucuronidase (Levvy *et al.* 1948), the effect of chloroform on the kidney-enzyme level in normal mice was seen to be confined to males. From the figures for total kidney glucuronidase in Table 1, it appears that the response in the male was abolished by castration 3 weeks previously, although this operation was not performed until the animals were adult. There was no necrosis, and Bowman's capsule had become predominantly female in character. In ovariectomized females, chloroform caused a small, but significant rise in the kidney enzyme, associated with necrosis and repair (for results one day after injection, $P = 0{\cdot}02$; grouping results for all three time intervals, $P < 0{\cdot}01$). Extending the period between ovariectomy and injection of chloroform from 3 to 13 weeks did not appreciably affect this response, but in the interval a change towards the male type of kidney became much more pronounced. As already noted, figures for uninjected mice showed no variations in *A* or *B*, corresponding to the changes in kidney morphology. The fact that in the male kidney the rise in glucuronidase appeared to be confined to fraction *B* may reflect an uneven distribution of the two fractions throughout this organ, with predominance of *B* in the convoluted tubules. In a histochemical study, Friedenwald & Becker (1948) found greater glucuronidase activity in rat-kidney tubules than in the glomeruli, when hydrolysis of suitable glucuronides in unfixed, frozen sections was allowed to proceed at pH 5.

The effects of sex hormones on liver and uterine glucuronidase

Fishman (1947) has examined the effects of testosterone propionate and oestradiol benzoate, separately and in combination, on uterine glucuronidase in ovariectomized mice. In the doses used, testosterone did not antagonize the action of the oestrogen in causing a rise in the enzyme level, and Fishman interpreted this as indicating 'a unique type of specificity of action by the oestrogen'. His results, however, show that administration of the androgen along with the oestrogen did not entirely prevent an increase in the wet weight of the uterus. By itself, testosterone produced a rise in glucuronidase activity and an increase in weight. Fishman's results seem entirely compatible with the view that an increase in glucuronidase activity in uterus, as elsewhere, reflects increased growth, and that his failure to observe antagonism between oestrogen and androgen resulted from use of too great an excess of the latter. Figures for uterus shown in Table 2 bear out this argument.

Oestrone and testosterone were given as single subcutaneous injections of the solutions in olive oil, alone or within 3 hr. of each other. Four days after injection of ovariectomized mice with 1·7 mg. oestrone/kg., uterine glucuronidase activity and weight were much greater than in untreated controls. The effects of 0·3 mg. oestrone/kg. and 3·3 mg. testosterone/kg. were alike in that there was a comparatively small rise in uterine weight, with a barely perceptible increase in the enzyme activity ($P = 0{\cdot}1$ and 0·02 respectively at the maximum activity). In a smaller dose (2 mg./kg.), testosterone had no action on the uterus, but this dose completely antagonized the effects of the larger dose of oestrone on the enzyme and the weight.

In the experiments with oestrone and testosterone, glucuronidase was determined in liver and kidney as well as in uterus. In ovariectomized mice a marked rise in liver glucuronidase, preceding that in uterus, was observed after injection of oestrone in a dose of 1·7 mg./kg. (Table 2). This effect was also seen in normal and castrate males, but was absent in intact females, even after 4·3 mg. oestrone/kg. Reducing the dose of oestrone to 0·3 mg./kg. abolished the action on liver in ovariectomized mice. Histological examination revealed intense mitotic activity, with little evidence of damage in the livers of oestrone-treated castrate males and females. In normal males, the effect on mitosis was slight, but there was a marked increase in binucleate cells. Testosterone, itself without any action on the liver, antagonized the stimulant effect of oestrone on the enzyme and on cell division. Bullough (1946) has studied the effects of oestrone on mitotic activity throughout the body of the adult female mouse, and concluded 'that those substances which have come to be called oestrogenic or female sex hormones are in fact general mitosis stimulators'. Oestrone produced no effect on liver in his experiments, which were, however, confined to the normal female.

Fractionation of the glucuronidase preparations was carried out in many of the experiments listed in Table 2. Fractions *A* and *B* were both involved in the liver response to oestrone, while all uterine activity was invariably found in fraction *A*.

The effects of liver regeneration on uterine enzyme and weight

Administration of carbon tetrachloride to rats has been shown to produce an increase in the weight of the uterus in immature animals (Talbot, 1939), and to enhance the effectiveness of administered oestrone in ovariectomized animals (Pincus & Martin, 1940). Partial hepatectomy causes a similar increase in the potency of administered oestrogen (Segaloff, 1946). From the work of Roberts & Szego (1947) it appears that increased sensitivity to oestrogens occurs during active liver regeneration rather than in the initial stages of injury. In all these studies the animals were treated with an oestrogen or alternatively the ovaries were still present.

It was, therefore, with surprise that increases in the β-glucuronidase activity and the weight of

Table 2. *Effects of oestrone and testosterone on liver, kidney and uterine glucuronidase*

(All values are given as mean ±S.E., followed (in parentheses) by the number of animals in the group.)

Treatment	Sex*	Days after treatment	Total G.U./g. tissue: Liver‡	Kidney‡	Uterus†	Uterine weight (mg.)
None	F.	—	334±48 (6)	266±39 (6)	333±53 (6)	234±56 (6)
	cF.	—	250±49 (6)	261±43 (6)	174±45 (6)	34±18 (6)
	M.	—	281±20 (6)	266±31 (6)	—	—
	cM.	—	301±18 (6)	286±20 (6)	—	—
Oestrone (4·3 mg./kg.)	F.	1	258±39 (3)	370±33 (3)	463±41 (3)	167±66 (3)
	F.	4	244±32 (3)	357±44 (3)	439±63 (3)	192±76 (3)
Oestrone (1·7 mg./kg.)	F.	1	365±60 (3)	223±47 (3)	388±45 (3)	320±24 (3)
	F.	4	267±26 (3)	253±41 (3)	441±87 (3)	253±31 (3)
	cF.	1	431±42 (3)	313±17 (3)	300±54 (3)	26±5 (3)
	cF.	2	481±34 (3)	275±67 (3)	343±47 (3)	47±8 (3)
	cF.	4	569±73 (6)	281±33 (6)	548±106 (6)	211±20 (6)
	cF.	6	399±67 (3)	356±52 (3)	346±41 (3)	52±10 (3)
	cF.	8	315±18 (3)	299±23 (3)	223±16 (3)	46±5 (3)
	M.	1	879±98 (9)	283±95 (9)	—	—
	M.	4	303±12 (6)	221±50 (6)	—	—
	cM.	1	359±43 (3)	324±22 (3)	—	—
	cM.	4	562±58 (3)	333±52 (3)	—	—
Oestrone (0·3 mg./kg.)	cF.	1	271±51 (3)	359±72 (3)	181±13 (3)	51±10 (3)
	cF.	2	274±34 (3)	321±30 (3)	247±38 (3)	47±2 (3)
	cF.	4	269±34 (3)	327±61 (3)	226±34 (3)	102±15 (3)
	cF.	6	256±38 (3)	305±22 (3)	181±36 (3)	63±9 (3)
Testosterone (3·3 mg./kg.)	cM.	1	271±50 (3)	172±14 (3)	—	—
	cM.	4	311±36 (3)	201±23 (3)	—	—
	F.	1	254±32 (3)	297±20 (3)	289±27 (3)	172±54 (3)
	F.	4	272±40 (3)	343±48 (3)	326±19 (3)	193±35 (3)
	cF.	0·5	236±62 (3)	339±38 (3)	199±20 (3)	57±12 (3)
	cF.	1	289±34 (3)	319±56 (3)	260±14 (3)	101±19 (3)
	cF.	2	284±25 (3)	363±46 (3)	183±35 (3)	44±6 (3)
	cF.	4	251±48 (3)	326±46 (3)	189±39 (3)	35±11 (3)
Testosterone (2 mg./kg.)	cF.	1	253±54 (3)	345±16 (3)	156±31 (3)	32±13 (3)
	cF.	4	259±18 (3)	345±25 (3)	154±31 (3)	39±9 (3)
Testosterone (2 mg./kg.) + oestrone (1·7 mg./kg.)	cF.	1	266±51 (6)	379±28 (6)	179±34 (6)	46±12 (6)
	cF.	4	279±20 (5)	340±56 (5)	205±31 (5)	47±7 (5)
	M.	1	261±20 (3)	314±31 (3)	—	—
	M.	4	295±78 (3)	323±64 (3)	—	—

*, †, ‡, see Table 1.

uterus were obtained 7 days after injection of ovariectomized mice with chloroform or carbon tetrachloride (Table 3). That these changes were not due to a direct action of the toxic agent on the uterus, but were secondary to the effect on liver, was shown by further experiments in which mice were submitted to partial hepatectomy 3 weeks after ovariectomy, with similar results. With all three methods of treatment, the rise in uterine weight at its greatest was statistically significant ($P < 0{\cdot}001$). Liver repair is far advanced after this period (Levvy *et al.* 1948). In a separate group of six mice, the uteri were examined histologically 9 days after partial hepatectomy. Metoestrus, pro-oestrus and, in one case, full oestrus were observed, as compared with dioestrus in ovariectomized controls. It should be noted that not more than 40 % of the liver was removed in the partial hepatectomies in the present experiments. Results obtained with mercuric nitrate suggest that changes in kidney are without effect on uterine weight and glucuronidase activity.

Fractionation of uterine-glucuronidase preparations from ovariectomized mice treated with chloroform and carbon tetrachloride showed all activity to be present in fraction *A*.

DISCUSSION

The original purpose behind the experiments described above was to decide whether the sites of action of various agents on glucuronidase activity were determined by differences in the properties of the enzyme. No evidence of this was obtained. An increase in β-glucuronidase activity appeared to be governed solely by the ability of the agent to stimulate growth processes in the organ in question. Results for uterus did, however, emphasize the need for a preliminary kinetic study with each new organ

Table 3. *Effect of liver regeneration on uterine enzyme and weight*

(All values are given as mean ±S.E., followed (in parentheses) by the number of animals in the group.)

Treatment	Sex*	Days after treatment	Total G.U./g. tissue			Uterine weight (mg.)
			Liver‡	Kidney‡	Uterus†	
None	F.	—	334±48 (6)	266±39 (6)	333±53 (6)	234±56 (6)
	cF.	—	250±49 (6)	261±43 (6)	174±45 (6)	34±18 (6)
Chloroform (2 g./kg.)	F.	1	812±91 (6)	227±23 (6)	265±45 (6)	277±44 (6)
	F.	4	696±61 (3)	238±27 (3)	342±34 (3)	372±36 (3)
	F.	7	705±64 (6)	310±55 (6)	321±51 (6)	306±29 (6)
	cF.	1	515±23 (3)	398±67 (6)	181±33 (3)	28±10 (3)
	cF.	4	620±26 (3)	352±24 (3)	162±31 (3)	33±7 (3)
	cF.	7	657±78 (5)	359±67 (7)	469±51 (7)	103±20 (7)
Chloroform§ (6 g./kg.)	cF.	8	605±75 (3)	435±56 (3)	386±61 (3)	99±18 (3)
	cF.	10	501±30 (3)	335±52 (3)	306±22 (3)	65±12 (3)
Carbon tetrachloride (5·3 g./kg.)	cF.	1	712±55 (3)	301±42 (3)	203±48 (3)	25±6 (3)
	cF.	4	664±57 (3)	235±23 (3)	285±52 (3)	18±9 (3)
	cF.	7	715±60 (6)	290±59 (6)	496±64 (6)	77±23 (6)
	cF.	10	379±56 (3)	292±12 (3)	205±20 (3)	42±5 (3)
Partial hepatectomy	cF.	2	572±53 (3)	284±22 (3)	225±67 (3)	75±14 (3)
	cF.	4	532±83 (3)	305±29 (3)	240±42 (3)	64±8 (3)
	cF.	6	625±89 (3)	342±34 (3)	412±31 (3)	123±14 (3)
	cF.	8	535±61 (6)	328±54 (6)	395±43 (6)	115±19 (6)
	cF.	12	424±41 (3)	329±32 (3)	277±29 (3)	53±6 (3)
Mercuric nitrate (20 mg./kg.)	cF.	3	241±32 (3)	523±27 (3)	179±18 (3)	30±4 (3)
	cF.	6	238±26 (3)	323±24 (3)	168±26 (3)	39±6 (3)

*, †, ‡, see Table 1.
§ Divided into three daily doses of 2 g./kg.; timed from first injection.

examined for glucuronidase activity. The choice of pH 5·2 for determining activities in liver or kidney with phenylglucuronide would seem to be justified for most purposes.

Changes in the susceptibility of the mouse kidney to chloroform necrosis were faithfully reflected in the figures for glucuronidase activity. The value of such figures as a biochemical index of growth is illustrated by the discovery of new facts relating to liver and uterus. The action of oestrone on liver in normal and castrate males and in ovariectomized females, and the antagonizing of this action by testosterone are in accordance with the view of Bullough (1946) that the effects of such hormones are more widespread throughout the body than is generally appreciated. The absence of any action by oestrone on liver in normal females suggests some form of control of this organ by the ovary.

The changes observed in uterus during liver regeneration in the ovariectomized mouse can only be explained on the assumption that the body is capable of producing an extra-ovarian growth hormone for uterus in significant amounts. In this case, there is obvious need for care in interpreting certain experiments (Talbot, 1939; Pincus & Martin, 1940; Segaloff, 1946; Roberts & Szego, 1947) in which the action of liver damage and regeneration in enhancing the effectiveness of administered or ovarian oestrogens is claimed to be due to 'depressed inactivation' or 'accelerated activation' of the hormones. In view of the effect of oestrone on the liver, a hitherto unsuspected complication must be looked for in the action of this compound on the uterus of the ovariectomized mouse.

SUMMARY

1. Both β-glucuronidase fractions found by Mills (1947) in ox spleen are present in mouse liver and kidney, whilst the uterus contains only one of these.

2. The two glucuronidase fractions in liver and kidney respond identically to agents causing changes in the enzyme activity.

3. In the uterus, as in other organs, changes in glucuronidase activity reflect changes in growth, and the action of oestrone on the enzyme is antagonized by testosterone.

4. Oestrone produces marked increases in glucuronidase activity and cell division in the liver in ovariectomized mice. This action, which is also seen in normal and castrate males, but not in normal females, is antagonized by testosterone.

5. During liver regeneration following chloroform or carbon tetrachloride poisoning or partial hepatectomy, uterine weight and glucuronidase activity increase in ovariectomized mice in absence of administered oestrogen.

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Concentration of Lipids in the Brain of Infants and Adults

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Histologically it has been shown that the fibre tracts of the brain are not fully myelinated at birth, but that myelination is completed later, coincident with the functional development of the central nervous system. Most of the medullated fibres of the nervous system are in the white matter, whereas most of the bodies of the nerve cells are in the grey matter. In order to investigate, therefore, the lipid components of the myelin sheath, the lipid distribution in the grey matter and white matter of the brain of the newborn infant has been compared with that of the adult brain.

The important lipids of the central nervous system are cerebrosides, cholesterol and the phospholipins, lecithin, sphingomyelin and kephalin. In a previous report (Johnson, McNabb & Rossiter, 1948*a*) it was shown that white matter of brain is distinguished from grey matter by a greater concentration of cerebroside, free cholesterol and sphingomyelin. It was suggested that these lipids, rather than lecithin and kephalin, formed the lipid components of 'myelin'. Additional evidence was obtained for this view when it was found that medullated peripheral nerve was relatively rich in cerebroside, cholesterol and sphingomyelin, thus resembling the white matter of brain rather than grey matter (Johnson, McNabb & Rossiter, 1948*b*). It has now been shown that white matter of adult brain, in which myelination is complete, differs from 'white matter' of newborn infant brain in that it contains a higher concentration of these same lipids (cerebroside, cholesterol and sphingomyelin).

METHODS

The brain was removed from each of five infants and five adults as soon as possible after death. The infants, whose ages ranged from 7 months' gestation (premature) to full term, died at birth or shortly afterwards. Samples of both grey matter and 'white' matter were taken from the cerebral hemispheres. The grey matter consisted of a thin shaving from the surface of the cerebrum. As there was practically no visible white matter in the infant, the 'white' sample was taken from the positions where white matter was known to occur in the adult brain.

A sample of tissue (1–3 g.) was rapidly weighed and repeatedly extracted with 50 ml. portions of a 1:1 ethanol-ether mixture as described previously (Johnson *et al.* 1948*a*). Additional samples of both grey matter and white matter were taken for the determination of the wet-weight to dry-weight ratio. A portion of the fresh tissue was added to a tared covered crucible, the crucible and tissue weighed, dried in an oven at 105° for 24 hr., cooled in a desiccator and re-weighed. From these figures, the water content of the tissue was calculated. The concentrations of cerebroside, free cholesterol, total cholesterol, total phospholipin, monoaminophospholipin and lecithin were determined in samples of the extract as previously described (Johnson *et al.* 1948*a*), and from these figures were calculated the concentrations of ester cholesterol, sphingomyelin and kephalin.

RESULTS

The figures for both white and grey matter of adult brain (Table 1) were similar to those obtained previously, when two human brains only were analyzed (Johnson *et al.* 1948*a*), and were considerably greater than those for infant brain, chiefly

Table 1. *Concentration of lipids in grey and white matter of infant and adult brain, expressed as percentage of fresh tissue weight*

(Five infant and five adult brains examined. Results given as mean ± S.E.M.)

	Grey matter			White matter		
	Infant (%)	Adult (%)	Adult/infant ratio	Infant (%)	Adult (%)	Adult/infant ratio
Cerebroside	0·52±0·06	0·87±0·24	1·67 : 1	0·58±0·01	4·78±0·19	8·25 : 1
Total cholesterol	0·49±0·06	0·99±0·02	2·02 : 1	0·67±0·07	4·22±0·17	6·30 : 1
Free cholesterol	0·48±0·07	0·97±0·05	1·98 : 1	0·64±0·07	4·15±0·18	6·50 : 1
Ester cholesterol	0·01±0·00	0·02±0·00	—	0·03±0·03	0·07±0·07	—
Total phospholipin	1·89±0·27	3·36±0·05	1·78 : 1	2·09±0·19	7·03±0·29	3·36 : 1
Monoaminophospholipin	1·77±0·26	2·83±0·08	1·60 : 1	1·96±0·20	5·03±0·37	2·57 : 1
Lecithin	0·75±0·08	0·99±0·09	1·32 : 1	0·85±0·07	1·37±0·12	1·61 : 1
Sphingomyelin	0·12±0·05	0·53±0·04	4·42 : 1	0·13±0·04	2·00±0·18	15·40 : 1
Kephalin	1·02±0·18	1·85±0·04	1·81 : 1	1·11±0·13	3·66±0·27	3·30 : 1

because the water content of adult brain was much less than that of infant brain.

Nevertheless, from Table 1, it can be seen that the ratio of the concentration of each individual lipid in the grey matter of the adult to the concentration of the same lipid in the infant was usually less than 2 and never greater than 4·42. For white matter the ratio for cerebroside, cholesterol (almost all of which was in the free form) and sphingomyelin was much greater, suggesting that it was a greater concentration of these lipids that distinguished the white matter of the adult brain from that of the infant.

The results of Table 1 appear somewhat different after allowance has been made for the water content of the tissues. Table 2 shows that the water content of infant brain was much higher than that of adult brain, and also that the value for grey matter of infant brain was not significantly different from that for white matter. The water content of the adult brain was much less, and it was found that grey matter had more than twice the water content of white matter. Thus, as the brain developed and myelin was deposited, the whole organ lost water, but relatively more was lost from the white matter than from the grey matter.

In Table 3 the values for the lipid components of both grey and white matter of infant and adult brain are given in terms of dry weight. It can now be seen how strikingly similar was the distribution of lipids in the grey matter of the infant brain to that in the adult. For grey matter, with the exception of sphingomyelin with a ratio of 2·33, the adult to infant ratio for each individual lipid was between 0·80 and 1·23. For white matter, however, the picture was quite different. The ratio for cerebroside and free cholesterol was 2·10 or more, and for sphingomyelin it was 4·87. Thus the white matter of adult brain had a higher concentration of cerebroside, free cholesterol and sphingomyelin than had the white matter of infant brain. The concentrations of total phospholipin in the white matter of the adult brain and infant brain did not differ significantly. There was no difference in the concentration of kephalin, and the greater concentration of sphingomyelin in the white

Table 2. *Water content of grey and white matter of infant and adult brain*

(Five infant and five adult brains examined. Results given as mean ±S.E.M.)

	Grey matter (mg./mg. dry tissue)	White matter (mg./mg. dry tissue)
Infant	9·74±0·69	9·82±0·72
Adult	5·34±0·10	2·40±0·06

Table 3. *Concentration of lipids in grey and white matter of infant and adult brain, expressed as percentage of dry tissue weight*

(Five infant and five adult brains examined. Results given as mean ±S.E.M.)

	Grey matter			White matter		
	Infant (%)	Adult (%)	Adult/infant ratio	Infant (%)	Adult (%)	Adult/infant ratio
Cerebroside	5·64±0·91	5·54±0·80	0·98 : 1	6·21±0·39	16·28±0·99	2·61 : 1
Total cholesterol	5·09±0·37	6·28±0·14	1·23 : 1	7·00±0·44	14·33±0·56	2·04 : 1
Free cholesterol	5·03±0·35	6·17±0·20	1·23 : 1	6·70±0·33	14·08±0·55	2·10 : 1
Ester cholesterol	0·06±0·04	0·10±0·05	—	0·30±0·07	0·26±0·22	—
Total phospholipin	19·56±1·39	21·27±0·49	1·08 : 1	22·04±0·59	23·84±0·73	1·08 : 1
Monoaminophospholipin	18·26±1·36	17·96±0·59	0·98 : 1	20·64±0·62	17·01±1·15	0·82 : 1
Lecithin	7·81±0·45	6·25±0·63	0·80 : 1	9·07±0·28	4·63±0·34	0·51 : 1
Sphingomyelin	1·30±0·46	3·03±0·29	2·33 : 1	1·40±0·58	6·82±0·67	4·87 : 1
Kephalin	10·46±1·08	11·71±0·40	1·11 : 1	11·57±0·58	12·39±0·83	1·07 : 1

matter of the adult brain was balanced by a lesser concentration of lecithin (Table 3). This point is well illustrated in Table 4, where the individual phospholipins are expressed as a percentage of the total phospholipin. For white matter the percentage of kephalin was about the same (52 %) for both infant and adult brain. Lecithin accounted for 41 % of the total phospholipin for infant brain and only 19 % for the adult, whereas sphingomyelin accounted for 6 % of the total phospholipin for infant brain and 29 % for the adult.

Table 4. *Concentration of phospholipins in grey and white matter of infant and adult brain, expressed as percentage of total phospholipid*

(Five infant and five adult brains examined. Results given as mean ±S.E.M.)

	Grey matter			White matter		
	Infant (%)	Adult (%)	Adult/infant ratio	Infant (%)	Adult (%)	Adult/infant ratio
Lecithin	40·28±2·31	29·16±2·46	0·72 : 1	41·22±1·85	19·42±1·35	0·46 : 1
Sphingomyelin	6·62±2·37	15·68±1·40	2·37 : 1	6·28±2·61	28·80±3·31	4·57 : 1
Kephalin	53·10±2·48	55·20±2·55	1·04 : 1	52·50±1·94	51·78±2·17	0·99 : 1

DISCUSSION

Frankel & Linnert (1910) found that the concentration of total lipid in the whole brain of the adult was greater than that in the brain of the newborn infant. Using the classical differential solubility techniques they showed that the increase in lipid concentration in adult brain was not confined to one lipid fraction only. This work was confirmed by Schiff & Stransky (1921) and recently by Schuwirth (1940). Raske (1886) found that the whole brain of foetal calves contained no cerebroside, a result confirmed by Mendel & Leavenworth (1908) using pigs. Noll (1899), when studying the 'protagon' fraction of human brain, observed that with the beginning of myelination there appeared an ethanol-soluble substance from which a reducing sugar was split off on hydrolysis. Koch & Koch (1913) showed that cerebroside was not present in the brain of newborn rats, but that the concentration of cerebroside, phospholipin and cholesterol increased with age. A similar finding was reported for the dog (Smith & Mair, 1912–13*a*), the rabbit (Backlin, 1930) and man (Koch & Mann, 1907; MacArthur & Doisy, 1919). Less comprehensive work on total phospholipin has been done for rat brain (Lang, 1937; Fries, Entenman, Changus & Chaikoff, 1941), and human brain (Bergamini, 1925; Singer & Deutschberger, 1928; Cattaneo, 1932). Similar studies on total cholesterol have been reported for rat brain (Lang, 1937; Fries *et al.* 1941), dog brain (Mansfeld & Liptak, 1913) and human brain (Rosenheim, 1914; Bergamini, 1925; Page & Menschick, 1931; Cattaneo, 1931). In addition, McConnell & Sinclair (1937) using rats, reported that the lecithin and kephalin fatty acids increased during growth. They did not estimate the individual phospholipins, nor did they distinguish between white and grey matter; hence from these studies very little can be deduced about the chemical nature of myelin.

That foetal brain contains more water than does adult brain was known to most of the above authors and the subject has been well reviewed for the rat by the numerous publications of Donaldson (see Donaldson & Hatai, 1931, for references). This raises the question of whether infant brain should be compared with adult brain on a wet-weight basis, as has been done by many workers. For instance, Backlin (1930) found that the concentration of total phospholipin in the whole brain of the adult rabbit was greater than that of a newborn rabbit in terms of wet weight, but that there was no difference when dry weight was used as a reference standard. We have found the same to be true for the white matter of human brain. In terms of wet weight (Table 1) the concentration of total phospholipin was greater in the adult than in the newborn infant, but when referred to unit dry weight there was no such difference (Table 3). Previously (Johnson *et al.* 1948*a*, *b*), lipids have been expressed as a percentage of 'essential lipid', i.e. as a percentage of the sum of the cerebroside, total cholesterol and total phospholipin. The values for the adult and infant brains were also calculated on an 'essential lipid' basis, but this method provided no further information than that already given in Table 3.

The recent report of Williams, Galbraith, Kaucher, Moyer, Richards & Macy (1945) on the lipids of the brains of rats of different ages is of interest for they estimated the individual phospholipins. It is difficult, however, to make a strict comparison of their results with our own, for, besides the species difference, the method which they used to distinguish the individual phospholipins was not the same as ours, and the white and grey matter were not separated. Williams *et al.* (1945) found that with increasing age, in addition to the well established greater concentrations of phospholipin, cerebroside and cholesterol, there was a large increase in kephalin, a lesser increase in sphingomyelin and a decrease in lecithin. In our study on the white matter of the human brain there was a lesser concentration of lecithin in the adult than in the infant, the same concentration of kephalin and a much greater concentration of sphingomyelin.

Many of the pioneers of brain chemistry noted the great difference between the lipids of white and those of grey matter. For instance, both Petrowsky (1873) and Thudichum (1901) found considerably more cholesterol and

cerebroside in white than in grey matter, and less lecithin. These findings have subsequently been confirmed (Smith & Mair, 1912–13*b*; Frankel & Linnert, 1910; Kirschbaum & Linnert, 1912; Yasuda, 1937; Randall, 1938; Johnson *et al.* 1948*a*). In addition, Schmidt, Benotti, Hershman & Thannhauser (1946) showed that the concentration of sphingomyelin in the white matter of ox brain was greater than that in grey matter. Many of these workers also noted that the concentration of water was greater in grey than in white matter.

That the lipids of white matter of foetal brain resemble those of grey matter was first pointed out by Raske (1886) and later by Frankel & Linnert (1910) and Smith & Mair (1912–13*b*). Not only have we confirmed this similarity for cerebroside, cholesterol and total phospholipin, but also for lecithin, sphingomyelin and kephalin.

Since the white matter of adult brain is distinguished from that of the brain of the newborn by greater concentrations of cerebroside, cholesterol and sphingomyelin, and, since it has been shown histologically that myelination of the various tracts is complete in the white matter of the adult brain and not in the brain of the newborn infant, it is perhaps reasonable to assume that these are the principal lipids of the myelin sheath. However, a word of caution is necessary. Waelsch, Sperry & Stoyanoff (1941) have pointed out that after birth lipid is deposited in the brain of the rat as a result of two processes, (*a*) growth and (*b*) myelination. Immediately after birth and before myelination is complete there is an active deposition of brain lipids as shown by isotope studies. The incorporation of radioactive phosphorus in the phospholipin fraction (Fries, Changus & Chaikoff, 1940) and of deuterium in both the fatty acid and non-saponifiable fraction of the brain lipids (Waelsch *et al.* 1941) was greatest in young rats immediately after birth, when growth was greatest, and decreased sharply with increasing age, even although myelination was proceeding rapidly. Such experiments show that much new lipid material is deposited in the brain after birth as a result of growth, and the differences between the adult and the newborn need not be necessarily the result of myelination.

In general, the lipid distribution is similar in both white and grey matter of infant brain and closely resembles that of grey matter of adult brain. The white matter of the adult is distinguished from that of the newborn by a much greater concentration of cerebroside, free cholesterol and sphingomyelin. This observation, together with the finding that these lipids are present in high concentrations in peripheral medullated nerves, makes it likely that cerebroside, free cholesterol and sphingomyelin, rather than lecithin and kephalin, go to make up the lipids of 'myelin'.

SUMMARY

1. The concentration of cerebroside, free cholesterol, total cholesterol, total phospholipin, lecithin, sphingomyelin and kephalin has been determined in both the grey and white matter of the brains of five infants and five adults.

2. The water content of both white and grey matter of infant brain was greater than that of adult brain, and for the adult the water content of grey matter was much greater than that of white matter.

3. The distribution of lipids in the white matter of infant brain resembled that of the grey matter and also the grey matter of adult brain.

4. The distribution of lipids in the white matter of adult brain differed from that of the white matter of infant brain in that there was a higher concentration of cerebroside, free cholesterol and sphingomyelin.

5. Although, in terms of dry weight, the white matter of adult brain had about the same total phospholipin content as white matter of infant brain, it contained more sphingomyelin and less lecithin.

6. The data reported here, taken in conjunction with those of previous studies, suggest that cerebroside, free cholesterol and sphingomyelin are the principal lipid components of the 'myelin' sheath of nerve fibres.

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Effects of Amidines on Oxidases of *Escherichia coli* and of Animal Tissues

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Interest in the diamidines as antiprotozoal and antibacterial agents led us to consider their possible mechanism of action, and a study of their effects on oxidation systems offered a method of approach. Bernheim (1943, 1944) reported that certain amidines inhibited the oxidation of D-proline and of both isomers of alanine by *Escherichia coli*, but were without effect on the oxidation of glucose, succinate or pyruvate at similar concentrations.

We considered that further valuable information would possibly be revealed by studying the effects of amidines at higher concentrations, using also a wider range of substrates. Accordingly, we have carried out an investigation along these lines, with *Esch. coli* as the test organism. Propamidine (1:3-di-(4′-amidinophenoxy)propane) was mainly employed, but a number of experiments were also carried out with hexamidine (1:6-di-(4′-amidinophenoxy)hexane) and two halogenated derivatives, i.e. dibromopropamidine (1:3-di-(2′-bromo-4′-amidinophenoxy)propane) and monoiodohexamidine (1-4′-amidinophenoxy-6-(2′-iodo-4′-amidinophenoxy)-hexane). Some parallel experiments on the oxidation systems of rat tissues were included. A recent communication (Wien, Harrison & Freeman, 1948), in which some of our results were quoted, has dealt with the diamidines as bactericides.

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EXPERIMENTAL

Measurement of respiration

The Warburg manometric technique was used for following O_2 uptakes at 37°. The bacterial suspension or tissue preparation was contained in the main vessel in 0·033 M-phosphate buffer (for tissue preparations, phosphate-Locke medium was used), with or without the amidine salt in appropriate concentration. A 6% KOH solution (0·2 ml.) was introduced into the central tube of the vessel, and soaked into a small piece of rolled Whatman no. 41 filter paper. The substrate solution (0·2 ml.) was contained in the side arm and was tipped into the main vessel after a suitable equilibration or incubation period. The total volume of vessel contents, including those of the side arm and central tube, was always 3 ml.

Organisms

Esch. coli (National Collection of Type Cultures no. 4144) was used. The organisms were sown on trypsin digest-agar plates, and after 18–24 hr. growth the cultures were washed off the plates, using 3 ml. of distilled water for each plate. The collected suspension was centrifuged, was twice washed by resuspending in distilled water and recentrifuging, and finally diluted to 7·15 mg. dry wt./ml. by the use of Wellcome standard opacity tubes. Of this suspension 0·5 ml. was used for each vessel. It was confirmed that no appreciable change in the suspension occurred during the time occupied by the experiments. The O_2 uptake due to the organisms alone, without substrate, was usually not greater than 20 μl./hr.; since this was small in comparison with the O_2 uptake in the presence of most substrates employed, it is permissible to regard the difference as representative of the O_2 used in the

oxidation of the substrate. Experiments were carried out at pH values of 5·6, 6·8, 7·4 and 7·8, in view of Bernheim's (1944) emphasis on pH.

Tissue preparations

Preparations were made from the organs of rats in the minimum possible time after killing.

Liver was ground with alundum and 3–4 ml. 0·9% NaCl to a fine suspension, filtered by squeezing through gauze, and the tissue washed by diluting to 10 ml. with 0·9% NaCl. After centrifuging at high speed the deposit was suspended in 0·9% NaCl (5 ml./g. original tissue) and 0·5 ml. used in each vessel for studying the activities of the succinate, choline and cytochrome oxidase systems.

Kidneys were minced in 0·9% NaCl to give a fine suspension, which was washed by centrifuging and diluted with 0·9% NaCl (5 ml./g. original tissue). For each vessel, 0·5 ml. was used for studying D-amino-acid oxidase activity.

Brain. The whole brain was made to a uniform suspension with 3–4 ml. 0·9% NaCl in a hard-glass homogenizer, and diluted with 0·9% NaCl (6 ml. homogenate/g. original tissue). Of this 1 ml. was used for each experiment with the lactate and glucose oxidase systems.

Amidine derivatives

These were used as double isethionate salts.

RESULTS

Effects of amidines on oxidases of Escherichia coli

The results of a series of experiments with a number of substrates at different pH values are shown in Table 1. No initial incubation period was allowed in these experiments, beyond that required for thermal equilibration. Fig. 1 illustrates the variations of oxygen uptake with time, in the case of lactate, and the manner in which this is influenced by propamidine at pH values of 5·6 and 7·8; similar variations with time were observed with the other substrates. The systems governing the oxidations of lactate, glucose, pyruvate, acetate and D-alanine were all substantially inhibited. The inhibitions were maximal after 2 hr. incubation at pH 7·8, and these effects may be compared with those observed by Bernheim (1944) with alanine and proline. With lactate, inhibitions of roughly the same order were brought about by dibromopropamidine, hexamidine and monoiodohexamidine.

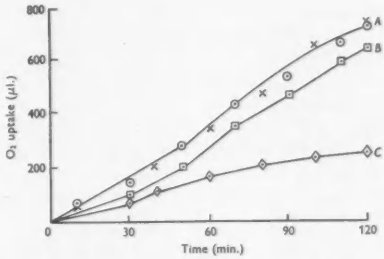

Fig. 1. Effects of propamidine on Na lactate oxidation by *Esch. coli*. Bacterial suspensions at 37° with 0·01 M-Na lactate. *A*, ⊙: pH 5·6. *A*, ×: with $12·5 \times 10^{-5}$ M-propamidine, pH 5·6. *B*: pH 7·8. *C*: with $12·5 \times 10^{-5}$ M-propamidine, pH 7·8.

Table 2 gives the results of a series of experiments in which the amidines were incubated with the bacterial suspension at pH 7·8 for 2 hr. at 37° before the addition of substrate; Q_{O_2} values (µl. O_2 absorbed/mg. dry wt. organisms/hr.) are included. Inhibitions by particular amidines on different substrates can be compared. Oxidation of all the substrates (malate, succinate and glutamate in addition to those mentioned in Table 1) were inhibited by concentrations of amidines of the order of $12·5 \times 10^{-5}$ M or less. Lactate was affected to approximately the same degree as alanine; both these systems were sensitive to concentrations of propamidine as low as $1·25 \times 10^{-5}$ M. The halogenated derivatives inhibited oxygen uptake slightly more than the parent compounds, and hexamidine rather more than propamidine, but the differences were small.

Table 1. *Effects of amidines on oxidations by* Escherichia coli

(Washed suspensions of bacteria, 37°; substrate added at the same time as amidine.)

		Inhibitions (%)							
		pH 5·6		pH 6·8		pH 7·4		pH 7·8	
Substrate (0·01 M)	Amidine ($12·5 \times 10^{-5}$ M)	In 1st hr.	In 2nd hr.	In 1st hr.	In 2nd hr.	In 1st hr.	In 2nd hr.	In 1st hr.	In 2nd hr.
Na lactate	Propamidine	8	9	23	35	38	57	39	78
Na lactate	Hexamidine	0	0	30	51	39	61	63	76
Na lactate	Monoiodohexamidine	25	24	45	52	66	84	73	90
Na pyruvate	Propamidine	0	0	0	19	30	32	33	21
Glucose	Propamidine	0	0	0	0	31	54	28	65
Na acetate	Propamidine	0	2	0	12	6	45	40	78
D-Alanine	Propamidine	15	44	25	50	21	59	53	73

Table 2. *Effects of amidines on oxidation systems of* Escherichia coli

(Washed suspensions of bacteria, incubated 2 hr. with amidine before tipping in substrate solution; reaction at 37°, pH 7·8.)

Substrate (0·01 M)	Amidine, concentration ($M \times 10^{-5}$)	Q_{O_2} Control without amidine	Q_{O_2} With amidine	Inhibition by amidine (%)
D-Alanine	Propamidine, 1·25	30·5	20·4	33
D-Alanine	Propamidine, 12·5	30·5	6·2	80
D-Alanine	Hexamidine, 12·5	33·2	2·0	94
D-Alanine	Dibromopropamidine, 12·5	33·2	4·2	87
Na lactate	Propamidine, 1·25	94·0	57·6	39
Na lactate	Propamidine, 12·5	94·0	30·0	68
Na lactate	Hexamidine, 12·5	94·6	22·4	76
Na lactate	Dibromopropamidine, 12·5	55·8	9·2	84
Na lactate	Monoiodohexamidine, 12·5	69·0	6·2	91
Glucose	Propamidine, 12·5	31·6	13·2	58
Glucose	Dibromopropamidine, 12·5	62·4	9·2	86
Glucose	Monoiodohexamidine, 12·5	77·0	0·6	99
Na malate	Propamidine, 12·5	26·2	7·2	72
Na malate	Hexamidine, 12·5	35·2	4·0	89
Na malate	Dibromopropamidine, 12·5	13·4	1·6	88
Na malate	Monoiodohexamidine, 12·5	39·6	3·0	92
Na acetate	Propamidine, 12·5	64·2	9·2	86
Na succinate	Propamidine, 12·5	34·6	20·0	42
Na glutamate	Propamidine, 12·5	14·6	4·4	69
Na pyruvate	Propamidine, 12·5	74·8	23·8	68

Table 3. *Effects of propamidine on tissue oxidation* (37° *and* pH 7·6)

Source of enzyme	Substrate, concentration (M)	Propamidine ($\times 12{\cdot}5 \times 10^{-5}$ M)	Period of incubation of enzyme and amidine (hr.)	Duration* of experiment (hr.)	O_2 uptake (μl.)	Q_{O_2}	Inhibition (%)
Rat-liver extract	Nil	0	2	1	2·7	0·06	—
	Na succinate, 0·03	0	2	1	333	8·32	—
	Na succinate, 0·03	1	2	1	313	7·82	6
	Choline chloride, 0·03	0	2	1	77	1·92	—
	Choline chloride, 0·03	1	2	1	23	0·58	73
	Choline chloride, 0·03	0	Nil	1	184	4·60	—
	Choline chloride, 0·03	1	Nil	1	48	1·20	74
	p-Phenylenediamine, 0·5	0	2	1	209	3·22	—
	p-Phenylenediamine, 0·5	1	2	1	233	5·84	0
Minced rat kidney	Nil	0	Nil	1	38	0·96	—
	D-Alanine, 0·013	0	Nil	1	169	4·22	—
	D-Alanine, 0·013	1	Nil	1	167	4·18	<1
Brain homogenate	Nil	0	1	2	66	0·50	—
	Na lactate, 0·017	0	1	2	165	1·23	—
	Na lactate, 0·017	1	1	2	149	1·11	10
	Glucose, 0·013	0	1	2	153	1·14	—
	Glucose, 0·013	1	1	2	131	0·97	14

* After tipping in substrate.

Effects of amidines on tissue oxidases

Effects of propamidine on some oxidation systems of rat tissues are shown in Table 3; the concentrations of the foregoing experiments were used. No significant effects were exerted on oxidation of glucose and lactate by rat brain; the succinate and cytochrome oxidases (the latter represented by the oxidation of *p*-phenylenediamine) and the D-amino-acid oxidase activity of kidney were likewise unaffected. The only positive effect noted was that on choline oxidase, which was appreciably inhibited by $12{\cdot}5 \times 10^{-5}$ M-propamidine.

DISCUSSION

The observed effects of the amidines on the oxidases of *Esch. coli* are of interest with reference to the antibacterial actions of these compounds. Although no definite conclusions regarding the mode of inhibition of bacterial growth may be drawn from results of

enzyme studies on only one organism, the ability of amidines to exert toxic effects on different oxidation systems is a factor to be taken into account, especially in view of the fact that a high pH value favours inhibition of growth (Elson, 1945) as well as of respiration. The connexion is not apparently a simple one, since the differences in bacteriostatic power between halogenated derivatives and parent compounds are not paralleled by the same differences in anti-oxidase activity. To give an example, the concentrations required to produce bacteriostasis for *Esch. coli* are $11{\cdot}8 \times 10^{-5}$M for propamidine and $0{\cdot}57 \times 10^{-5}$M for dibromopropamidine (Wien *et al.* 1948): the corresponding figures for inhibition of oxidation were 80 and 87 % for alanine, and 68 and 84 % for lactate, using $12{\cdot}5 \times 10^{-5}$M concentrations of the amidines. It cannot, therefore, be claimed that the anti-oxidase effects reflect the therapeutic efficacy of the compounds.

Regarding the mechanism of the inhibitory effect on respiration, it may be that the amidines exert effects on the particular dehydrogenases concerned with the different substrates, but the fact that so many systems are involved would appear to render this unlikely. It is more likely that an inhibitory effect is exerted on a respiratory mediator common to the various systems. One interesting possibility is that the system governing the direct transfer of molecular oxygen may be depressed; this would partly be supported by the absence of effect on the lactate oxidation system of brain, a cozymase-linked system, and by the slight effects of the amidines in inhibiting the growth of anaerobic organisms. It is conceivable that the cytochrome system peculiar to *Esch. coli* may be inhibited; the absence of an effect on the cytochrome oxidase of liver does not conflict with this possibility, in view of the different cytochrome compositions of the two types of cell (Keilin & Harpley, 1941). It was not possible to pursue the point.

Finally, experiments on tissue oxidation revealed an apparently specific inhibition of the choline oxidase of liver. This point is of interest in connexion with the chronic toxicity of the amidines, which produce characteristic fatty changes in the liver. In view of the part played by choline metabolism in liver function, especially in regard to fatty changes, the inhibitory effect of propamidine on choline oxidase *in vitro* may well have some bearing on the degenerative changes produced in the liver *in vivo*.

SUMMARY

1. The respiration of washed cells of *Escherichia coli* in presence of various substrates was strongly inhibited by certain diamidines in concentrations of the order of $1{\cdot}25$–$12{\cdot}5 \times 10^{-5}$M.

2. There was no simple relationship between the bacteriostatic potencies of the amidines and their effects on respiration.

3. Propamidine had no effect on several tissue oxidation systems, but inhibited the choline oxidase of liver.

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The Quantitative Determination of Barbiturates in Tissues by Ultraviolet Absorption Spectrophotometry

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The purpose of this paper is to describe a method for the estimation of very small amounts of barbiturates in mammalian tissues. The problem arose in connexion with work on the intermediary metabolism of the drugs, when it was found that the methods previously described were inapplicable for various reasons which will be discussed.

The basis for the various spectrophotometric procedures is the fact that barbituric acid and its derivatives of pharmacological importance, including

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barbital (5:5-diethylbarbituric acid) and pentobarbital (5-ethyl-5-2′-pentylbarbituric acid) possess in alkaline solution characteristic absorption bands in the ultraviolet. Changes in the location and intensity of these bands in relation to variations in molecular structure have been discussed by Loofbourow & Stimson (1940), Stuckey (1940, 1941, 1942), Klotz & Askounis (1947), and Walker, Fisher & McHugh (1948). The facts relevant to the present method are: (*a*) from *c.* pH 6·5–*c.* pH 10, a well-defined absorption maximum occurs at a wave length of 239 mμ.; (*b*) as the reaction becomes still more alkaline, the position of the maximum shifts in the direction of longer wave lengths; (*c*) in alkaline solution, the extinction at 239 mμ. is directly proportional to the concentration of barbiturate, in accordance with the Lambert-Beer law (Fig. 1). On the basis, therefore, of spectrophotometric data the concentration of the drugs can easily be determined in pure solutions.

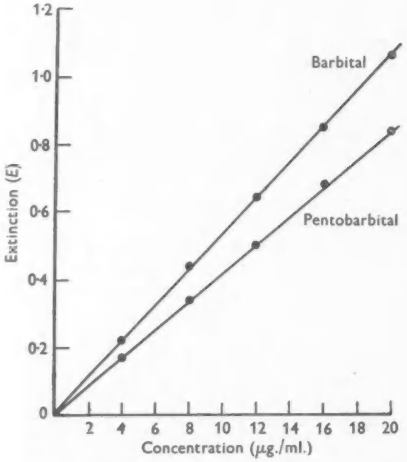

Fig. 1. Relation of extinction to concentrations of barbital and pentobarbital in alkaline solutions; pH 9·4; wave length 239 mμ.

In attempts made to recover barbiturate from tissues, however, other substances are usually extracted at the same time. Some of them absorb radiation in the same region of the ultraviolet as the barbiturates. It has not been practicable to recover the drugs quantitatively in 'pure' solution, and it is therefore evident that any spectrophotometric method for the estimation of barbiturate in the presence of such impurities is valueless unless it allows for the contribution made by these interfering substances to the total absorption.

The present method achieves this by making use of the observation that the intensities of absorption at 239 mμ. of barbiturate and of the interfering substances differ in their dependence on pH. The extinction of barbiturate is characteristically related to pH, being maximal at pH 10 and decreasing steeply towards the acid, less steeply towards the alkaline side (Fig. 2). The ratio of the extinctions at any two values of pH is independent of concentration.

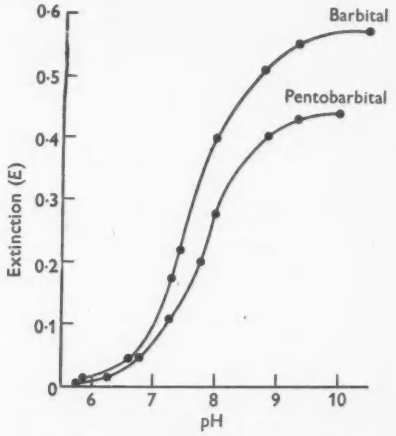

Fig. 2. Relation of extinction to pH for barbital and pentobarbital; concentration of drugs 10 μg./ml.; wave length 239 mμ.

In contrast, the extinction due to the interfering substances obtained from different tissues by the present procedure has been shown to be practically constant over certain ranges of pH. Preliminary experiments are necessary to determine these ranges and the change in extinction of barbiturate when the pH is altered to the same extent. The results permit calculation of the concentration of barbiturate in impure solutions of this nature.

METHOD

Principle. The tissue is homogenized and the proteins are precipitated by ethanol. The barbiturate in the acidified protein-free filtrate is extracted with ether and passed into alkali. To two samples of this alkaline solution are added phosphate or borate solutions which bring the pH to different known values. The extinctions at 239 mμ. of the resulting solutions are measured, and from these the concentration of barbiturate in the tissue can be determined by calculation.

Apparatus. The extinction measurements were made with a Beckman Model DU photoelectric spectrophotometer with 1 cm. quartz cells. pH was determined electrometrically with a glass electrode.

Reagents. Conc. HCl (A.R.); absolute ethanol; diethyl ether (A.R.); NaCl (A.R.); NaOH solution 0·2M; KH_2PO_4 and H_3BO_3-KCl solutions as for the preparation of buffer solutions according to Vogel (1944), except that the concentrations of the stock phosphate and borate solutions are 1·0M and 0·5M respectively.

Experimental procedure

Preliminary experiments. About 1 g. of normal tissue is extracted as described below. The extracted interfering substances are highly concentrated in the 5 ml. of alkaline solution obtained; this is therefore made up to 50 ml. with more 0·2M-NaOH solution, and 5 ml. amounts of this are then pipetted into a series of test tubes graduated at 10 ml.

Into another series of test tubes similarly graduated are put 5 ml. volumes of a 0·002% (w/v) solution of the barbiturate in 0·2M-aqueous NaOH (equivalent to 100 μg. drug/tube). Into a third series of test tubes are put 5 ml. volumes of pure 0·2M-NaOH solution.

To corresponding tubes of all three series are added increasing volumes of the phosphate or borate solutions, so that the pH increases in each series by steps of approx. 0·5 of a unit from 6 to 10. The quantities to be added may be calculated from the tables given by Vogel (1944). The solutions are then made up to 10 ml. with water.

The extinctions at 239 mμ. of the first two series of solutions are measured, using the third series as blanks. Then the pH of each solution in the first two series is accurately determined. Curves are plotted to show extinction-pH relationships. These curves show the pH range over which the extinction of the interfering substances is constant, and they show the constants K and k (defined below).

Extraction. The tissues are thoroughly homogenized (Potter & Elvehjem, 1936). The homogenate is transferred quantitatively into a narrow glass-stoppered measuring cylinder. Absolute ethanol (2 vol.) is added and the mixture is shaken and allowed to stand for 15 min. Water ($\frac{1}{2}$ vol.) is added and the volume of the resulting solution noted. The mixture is then filtered through a Whatman no. 42 paper. The clear filtrate is saturated with NaCl. One half of it is taken into a separating funnel, acidified with a drop of concentrated HCl, and extracted once with an equal volume of ether.

The aqueous layer is discarded. The ethereal layer is extracted once, in the same separating funnel, with approx. 5 ml. of 0·2M-NaOH. The alkaline layer is run off, and most of the ether is removed by a current of N_2. The solution is then made up to 10 ml. with water.

Spectrophotometry. Portions of this alkaline solution (5 ml.) are placed in each of two test tubes graduated at 10 ml. The pH values of these two samples are adjusted to the different desired values by the addition of appropriate volumes of phosphate or borate solutions; the volumes are adjusted to 10 ml. with water. The extinctions at 239 mμ. are then determined.

Calculation. The two solutions submitted to spectrophotometry are designated S_1 and S_2, and their hydrogen ion concentrations pH_1 and pH_2 respectively, where $pH_1 > pH_2$. The following symbols are also used:

R_1 = Extinction of S_1.
R_2 = Extinction of S_2.
x_1 = Extinction of impurities in S_1.
x_2 = Extinction of impurities in S_2.
K = Ratio of extinctions at 239 mμ. of the barbiturate at pH_1 and pH_2. This is a constant, the value of which is found by reference to curves obtained from the preliminary experiments (e.g. Fig. 2).
k = Ratio of extinctions at 239 mμ. of the barbiturate at pH_1 and pH 10. This is also a constant and its value is obtained from the same curves.
M = Mol. wt. of the barbiturate (Table 1).
$\epsilon_{max.}$ = Molecular extinction at 239 mμ. of the barbiturate at pH 10 (Table 1).
W = Weight of tissue (g.)

First, the extinction of the impurities in S_1 and S_2 must be calculated. If, as is usually the case, $x_1 = x_2$, it is clear that

$$K = R_1 - x_1/R_2 - x_1. \quad (1)$$

Therefore

$$x_1 = KR_2 - R_1/K - 1. \quad (2)$$

The concentration of barbiturate in S_1 and S_2 is

$$(kR_1 - x_1)\frac{10^3 M}{\epsilon_{max.}}\ \mu g./ml. = \left[kR_1 - \frac{KR_2 - R_1}{K - 1}\right]\frac{10^3 M}{\epsilon_{max.}}\ \mu g./ml. \quad (3)$$

In the procedure suggested the tissue barbiturate is diluted 1 in 40; therefore the concentration of barbiturate in the tissue is

$$\left[kR_1 - \frac{KR_2 - R_1}{K - 1}\right]\frac{4 \times 10^4 M}{W\epsilon_{max.}}\ \mu g./g. \quad (4)$$

This formula can be simplified if it can be shown that the extinction due to the impurities does not change significantly when the pH is varied even more, i.e. from 10 to 6·5 or lower. At pH 10 barbiturates absorb maximally; below pH 6·5 they cease to absorb altogether. Therefore (3) reduces to

$$(R_1 - R_2)\ 10^3 M/\epsilon_{max.}\ \mu g./ml., \quad (5)$$

and (4) to

$$(R_1 - R_2)\ 4 \times 10^4 M/W\epsilon_{max.}\ \mu g./g. \quad (6)$$

If the extinction due to the impurities does not remain absolutely constant between pH_1 and pH_2, but shows small, determinable, variations, it is possible to correct the error introduced thereby, as follows:

If $x_2 = nx_1$ (1) may be written

$$K = R_1 - x_1/R_2 - nx_1, \quad (7)$$

and

$$x_1 = KR_2 - R_1/nK - 1. \quad (8)$$

Therefore, (4) becomes

$$\left[kR_1 - \frac{KR_2 - R_1}{nK - 1}\right]\frac{4 \times 10^4 M}{W\epsilon_{max.}}\ \mu g./g. \quad (9)$$

and (6) becomes

$$(R_1 - R_2/n)\ 4 \times 10^4 M/W\epsilon_{max.}\ \mu g./g. \quad (10)$$

Table 1. *Molecular weights and molecular extinctions of barbital and pentobarbital*

Compound	Mol. wt. (M)	Concentration (μg./ml.)	Concentration (mM/l.)	Observed extinction at 239 mμ. and pH 10	Molecular extinction coefficient $\epsilon_{max.}$
Barbital	184·2	10	$0{\cdot}054_3$	0·570	10,500
Pentobarbital	226·3	10	$0{\cdot}044_1$	0·441	10,000

Table 2. *Experimental data on solutions examined*

(Buffers added: S_1, borate (0·5M); S_2, phosphate (1·0M).)

	S_1			S_2				
Tissue extracted wet wt. (300–500 mg.)	Vol. of buffer added ml.	pH_1	Extinction observed (E, 1 cm.)	Vol. of buffer added ml.	pH_2	Extinction observed (E, 1 cm.)	K	k
Rat liver	2·3	10·0	0·184	1·06	8·0	0·180	—	—
Rat liver	2·3	10·0	0·116	1·06	8·0	0·119	—	—
Rat kidney	2·3	10·0	0·126	3·97	6·4	0·128	—	—
Rat-heart muscle	2·3	10·0	0·104	3·97	6·4	0·102	—	—
Barbital	2·3	10·0	0·572	1·06	8·0	0·377	1·52	1
Pentobarbital	2·3	10·0	0·440	1·06	8·0	0·258	1·71	1

RESULTS

Known amounts of barbital and pentobarbital were added to pieces of rat tissue, re-extracted and estimated according to the method described. Table 2 shows experimental data and Table 3 the results of recovery experiments. It will be seen that recoveries were satisfactorily constant and close to 100%.

Table 3. *Recovery of barbital and pentobarbital added to various tissues*

Tissue (wet wt. 300–500 mg.)	Drug added	Wt. added (μg.)	Recovery (%)
Rat liver	Barbital	200	95
	Barbital	100	90
	Pentobarbital	200	95
	Pentobarbital	100	94
Rat kidney	Barbital	200	91
	Pentobarbital	200	84
Rat-heart muscle	Barbital	200	92
	Pentobarbital	200	99

DISCUSSION

Published procedures for the estimation of very small amounts of barbiturates are based either on the cobalt colour reaction or on ultraviolet absorption spectrophotometry.

The cobalt colour reaction (Koppanyi, Murphy & Krop, 1933; Koppanyi, Dille, Murphy & Krop, 1934) depends upon the formation of a reddish colour when barbiturates in chloroform solution are treated with a cobalt salt in the presence of *iso*propylamine or some other weakly alkaline reagent. The test has been repeatedly criticized for its lack of specificity (Riley, Krause, Steadman, Hunter & Hodge, 1940) and for its relative insensitivity. It was originally claimed that the reaction was sensitive to barbital in concentrations of 10 μg./ml. of the chloroform solution used for colorimetry, but Cohen (1946) found that the range of concentrations susceptible to accurate estimation was 1–10 mg./ml. The author has found that the colorimetric estimation of concentrations smaller than 0·1 mg./ml. is difficult and inaccurate; Hellman, Shettles & Stran (1943) stress the fact that the pink colour obtained is transient and difficult to read.

Ultraviolet spectrophotometry has formed the basis of a number of earlier methods for the quantitative determination of micro amounts of barbiturates. The technique of Hellman *et al.* (1943) is limited to thiobarbiturates. Dorfman & Goldbaum (1947) reported experiments with barbiturates and referred to a spectrophotometric method (Jailer & Goldbaum, 1946; Goldbaum & Hildalgo, 1949), but no detailed description of their procedure, other than that applicable to pentothal, is available at the time of writing.

Very recently, and unknown to the author until after the completion of the experimental work, Walker *et al.* (1948) have described a spectrophotometric method which is based on considerations similar to those underlying the method described in this paper. These workers, after measuring the extinction in aqueous NaOH solution at pH 10, due to barbiturate and to interfering substances, abolish the extinction due to barbiturate by acidification with a micro drop of sulphuric acid, and again measure the extinction. They find that the extinction due to interfering substances extracted from blood does not alter appreciably between pH 10 and a strongly acid reaction, and they are thus able to calculate barbiturate concentration from the simple difference in the extinctions measured. Their formula is, therefore, essentially the same as equation (5) above. They suggest that this method might also prove applicable to tissues other than blood. The present author has not been able to confirm this suggestion in the case of liver extracts; indeed he has observed that the extinctions of such extracts, obtained and treated in a manner closely similar to that of Walker *et al.* (1948), show variations of up to 20%. Only over a relatively narrow range of pH have the extinctions been found constant to within the experimental error. Another difficulty

referred to by Walker *et al.* (1948) is that not infrequently acidification with strong acid brings about clouding of the solution which is thereby rendered useless for further absorptiometry. This has not occurred in our procedure. Moreover, the recoveries obtained by the method of Walker *et al.* (1948) are low, the average being 70%.

Ultraviolet spectrophotometry permits the estimation of concentrations of barbiturates as low as 1–2 μg./ml. in pure solution. Since the sensitivity of a method based on ultraviolet spectrophotometry is limited by the ratio of the extinction by barbiturate to the extinction by interfering matter, attempts have been made to obtain the drugs from tissue extracts in pure solution. Earlier procedures employed charcoal for clearing the extracts (Delmonico, 1939, 1940; Levvy, 1940; Anderson & Essex, 1942), but charcoal adsorbs significant amounts of barbiturate (Hellman *et al.* 1943). Hellman and coworkers washed ethereal extracts of the drug from blood with 0·5M-sodium bicarbonate, but according to Walker *et al.* (1948) this again causes the loss of some of the drug. Purification of the drugs by chromatography on alumina columns was described by Raventos (1946), and their isolation by sublimation was used by Cohen (1946). Various micro sublimation procedures were tried here, but it was not found possible to ensure purification and quantitative recovery simultaneously.

The present method makes a high degree of purification unnecessary, since it permits accurate estimation of barbiturate concentrations in the presence of unknown impurities. In particular, it has the following advantages:

(1) Barbiturates are highly soluble in 50% ethanol, whether the reaction is neutral, acidic or basic. Ethanol extraction appears to promote full recovery of the drugs without increasing the amount of impurities extracted.

(2) Absorption spectrophotometry is affected neither by traces of ether left in solution, nor by the variations in the concentrations of the buffering agents, provided use is made of corresponding blank solutions.

(3) The method has been found to be applicable to blood, naturally without preliminary homogenization, and with the following alterations in the procedure for protein precipitation: To 1 vol. of whole blood in a boiling tube is added one fifth vol. of molar potassium dihydrogen phosphate which brings the pH to between 4·5 and 5·0. The tube is heated on a boiling water bath for 3 min. After cooling, 2 vol. absolute ethanol and 1 vol. water are added, the mixture shaken and filtered and the procedure is then continued as before.

SUMMARY

1. A procedure based on ultraviolet spectrophotometry is described for the quantitative determination of very small amounts of barbiturates in tissues and blood.

2. Complete elimination of contaminants is not attempted. The difficulty due to the simultaneous extraction of barbiturate and impurities absorbing in the same region of the ultraviolet is overcome by the use of differential spectrophotometry, depending on extinction-pH relationships.

3. The procedure is rapid, accurate and sensitive and appears to offer some advantages over methods previously described.

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Nicotinamide Biosynthesis by Intestinal Bacteria as Influenced by Methyltryptophans

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In recent papers (Ellinger & Abdel Kader, 1947, 1948, 1949*b*) it has been shown that *Bacterium coli* (*Escherichia coli*) was able to synthesize nicotinamide from ornithine, but not from tryptophan, and that mixed cultures from faeces or caecum contents could synthesize nicotinamide also from tryptophan. It was suggested that in the conversion of tryptophan to nicotinamide, ornithine was formed as intermediate by a cleavage of the tryptophan molecule. It was hoped that the utilization by the intestinal flora of methyltryptophans with the methyl group in various positions, i.e. in the alanine side chain, in the pyrrole and in the benzene nucleus, might throw some light on this theory. This was not so for reasons to be discussed later, but the experiments provided other results which are to be presented in this paper.

METHODS

The experiments were carried out *in vitro* with mixed cultures from rat caecum contents, consisting mainly of coliform bacteria, staphylococci, *Streptococcus faecalis*, plenty of an unidentified coccus growing in very small colonies and pure cultures of *Bact. coli* 4*c* (Type I faecal; Ministry of Health, 1939). The technique used was mainly that described before (Ellinger & Abdel Kader, 1949*b*). Growth was measured by assessing opacity with the Brown scale.

The following compounds were tested: DL-ornithine, DL-tryptophan, DL-2-methyl-, DL-4-methyl-, DL-5-methyl-, and DL-7-methyl-tryptophans,* and L-α-methylamino-β-(3-indolyl) propionic acid (abrin). The 2-, 4-, 5- and 7-methyltryptophans were kindly supplied by Dr H. N. Rydon who described their preparation and properties (Rydon, 1948). Abrin was prepared from the seeds of *Abrus praecatorius* by a method similar to that of Hoshino (1935). The seeds were cracked and the husks removed; the cotyledons were then ground to a fine powder, dried *in vacuo* over P_2O_5 and extracted with ether to remove as much as possible of a yellow oil which interferes with the precipitation of the abrin. The powder was filtered off and extracted to exhaustion with methanol in a shaking machine at room temperature (5–6 hr.). The powder was filtered off and washed with methanol. Filtrate and washings were evaporated to dryness at reduced pressure. When the dry residue was washed with a little cold water the abrin separated as a white powder which was purified by further washings with cold water on the centrifuge. It was then recrystallized several times from hot water, m.p. 292° (decomp.)

Media of ammonium lactate (Fildes, 1938) containing one of the compounds mentioned in 2 mM concentration were incubated with suspensions of mixed cultures of caecum contents of rats. In another series the same media containing ornithine or tryptophan alone, or ornithine plus either tryptophan or one of the various methyltryptophans, were incubated with a pure culture of *Bact. coli* 4*c*. In a third series ammonium lactate containing ornithine and one of the five methyltryptophans in concentrations from 0·01 to 2 mM was incubated with *Bact. coli* 4*c* for 72 hr. In all three series growth and nicotinamide formation were estimated as before (Ellinger & Abdel Kader, 1949*b*).

The effect of the methyltryptophans on acid production by *Lactobacillus arabinosus* had to be tested, since it was the basis for the assay of nicotinamide. For this purpose the Barton-Wright (1944) media containing one of the methyltryptophans in 2 mM concentration, the concentration present in the assay, were inoculated with a suspension of *Lb. arabinosus* and the acid formed was estimated after incubation for 72 hr. at 37°.

In order to obtain information about the effect of the different methyltryptophans on the growth of the various intestinal bacteria, agar plates were inoculated with equal amounts of the 24 hr. cultures using standardized Pasteur pipettes and incubated for 48 hr. Separate counts were made for the most frequent bacteria. They were compared with similarly made plates from cultures in pure ammonium lactate.

RESULTS

In pure cultures of *Bact. coli* 4*c* growth was very little affected by any of the compounds tested (Table 1). Nicotinamide formation from ammonium lactate was slightly inhibited by tryptophan and increased in the usual way by ornithine (Ellinger & Abdel Kader, 1949*b*). The nicotinamide formation from ornithine was slightly inhibited (by 6%) by tryptophan and by abrin (by 14%) and completely inhibited by 2-, 4-, 5- and 7-methyltryptophans in 2 mM concentration. In smaller concentrations (Table 2) growth was very little affected, and, if so, slightly increased; only once a slight inhibition (by 5%) was observed with one concentration of the 7-methyl derivative; this was within the limits of error of the method. Nicotinamide formation was inhibited to different degrees, rising with the concentrations of 2-, 4-, 5- and 7-methyltryptophans, and hardly inhibited by abrin.

In the experiments (Table 3) using mixed cultures from the caecum content, growth was not markedly affected by any of the compounds tested. The viable counts from 24 hr. cultures showed an undiminished growth of coliforms. Nicotinamide production was stimulated by ornithine and tryptophan and by

* The numbers 2, 4, 5 and 7 refer to the positions in the indole nucleus, the usual convention being followed.

Table 1. *Effect of ornithine, tryptophan and ornithine plus tryptophan or one of five methyltryptophans on growth and nicotinamide formation by* Bacterium coli 4c *in ammonium lactate medium*

Compound tested (2 mM)	Growth (% of control)	Nicotinamide formed (mμg./ml. medium)	Nicotinamide formed (% of ornithine control)
None	100	10	29
DL-Ornithine	100	35	100
DL-Tryptophan	100	8	23
DL-Ornithine + DL-tryptophan	100	33	94
DL-Ornithine + DL-2-methyltryptophan	100	0	0
DL-Ornithine + DL-4-methyltryptophan	100	0	0
DL-Ornithine + DL-5-methyltryptophan	100	0	0
DL-Ornithine + DL-7-methyltryptophan	100	0	0
DL-Ornithine + L-abrin	100	30	86

Cell content of inoculum: about 6×10^5/ml.; of cultures after 48 hr. growth, about 380×10^6/ml.

Table 2. *Effect of various concentrations of* 2-, 4-, 5- *and* 7-*methyltryptophans and of abrin, on growth and nicotinamide formation by* Bacterium coli 4c *in ornithine-ammonium lactate medium*

Concentrations of methyltryptophans (mM)	2-Methyltryptophan			4-Methyltryptophan		
	Growth (% of control)	Nicotinamide formed (mμg./ml.)	Nicotinamide formed (% of control)	Growth (% of control)	Nicotinamide formed (mμg./ml.)	Nicotinamide formed (% of control)
0	100	29	100	100	26	100
0·01	125	20	69	100	18	69
0·03	125	20	69	100	15	58
0·1	125	15	52	100	14	54
0·3	125	6	21	100	6	23
1·0	100	25	9	100	5	19
2·0	100	0	0	100	0	0

Concentrations of methyltryptophans (mM)	5-Methyltryptophan			7-Methyltryptophan			Abrin		
	Growth (% of control)	Nicotinamide formed (mμg./ml.)	Nicotinamide formed (% of control)	Growth (% of control)	Nicotinamide formed (mμg./ml.)	Nicotinamide formed (% of control)	Growth (% of control)	Nicotinamide formed (mμg./ml.)	Nicotinamide formed (% of control)
0	100	29	100	100	26	100	100	29	100
0·01	125	28	96	100	14	54	100	29	100
0·03	125	27	93	100	13	50	100	29	100
0·1	125	23	79	100	6	23	150	28	96
0·3	125	14	48	100	3·7	14	150	27	93
1·0	100	6	21	95	2·5	10	100	26	89
2·0	100	0	0	100	0	0	100	25	86

Inoculum for 2- and 5-methyltryptophans and for abrin, 62×10^4 cells; and for 4- and 7-methyltryptophans 61×10^4 cells; growth of control after 72 hr., 76×10^6 cells/ml.

Another similar experiment gave similar results.

Table 3. *Effect of ornithine, tryptophan and five methyltryptophans on growth and nicotinamide formation by mixed cultures from rat caecum contents in ammonium lactate medium*

Compound tested (2 mM)	Growth (% of control)		Nicotinamide formed (mμg./ml. medium)		Nicotinamide formed (% of control)	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
None	100	100	10	6	100	100
DL-Ornithine	100	64	22	56	220	933
DL-Tryptophan	100	100	18	27	184	450
DL-2-Methyltryptophan	100	100	6	0	60	0
DL-4-Methyltryptophan	100	100	6	0	60	0
DL-5-Methyltryptophan	155	100	6	0	60	0
DL-7-Methyltryptophan	155	100	8	0	80	0
L-Abrin	100	100	19	29	186	483

Four more experiments showed complete inhibition in one case and partial inhibition in three cases by 2-, 4-, 5- and 7-methyltryptophans.

abrin to the same extent as by tryptophan. In Exp. 1, Table 3, it was inhibited to a considerable but varying degree, and in Exp. 2 completely, by the 2-, 4-, 5- and 7-methyltryptophans in concentrations of 2 mM.

Table 4. *Effect of the five methyltryptophans on the acid production by* Lactobacillus arabinosus *in Barton-Wright medium*

Compound tested (2 mM)	Acid produced/ml. medium (ml. 0·1 N-NaOH)	(% of control)
Control	0·672	100
DL-2-Methyltryptophan	0·680	101
DL-4-Methyltryptophan	0·684	102
DL-5-Methyltryptophan	0·684	102
DL-7-Methyltryptophan	0·254	37
L-Abrin	0·692	103

Growth of *Lb. arabinosus* in the Barton-Wright medium was inhibited by 7-methyltryptophan and unaffected by the others (Table 4). This allows the use of the bacterium for quantitative assay for all but the 7-methyl derivatives. Since the inhibition of *Lb. arabinosus* by this compound was incomplete, it could be concluded that the 7-methyltryptophan also interfered with the nicotinamide synthesis by *Bact. coli*.

The viable counts of the mixed cultures from the caecum contents did not show any marked diminution of the main representatives of the intestinal flora by any of the methyltryptophans in 2 mM concentration.

DISCUSSION

Inhibition of growth by the various methyltryptophans with methyl groups in the indole nucleus has been observed for *Bacterium typhosum*, for which tryptophan is an essential nutrient, by Fildes & Rydon (1947). *Bact. coli* does not need tryptophan for growth, and, as would be expected, the methyltryptophans in concentrations up to 2 mM did not interfere markedly with the growth of *Bact. coli*, although Anderson (1945) observed complete inhibition of growth of a certain strain by 5-methyltryptophan (*Bz*-3-methyltryptophan) in concentrations of 18 μM. The difference might be due to the strain, the nature of which is not described by Anderson. The non-utilization of the 2-, 4-, 5- and 7-methyltryptophans by *Bact. coli* made it impossible to draw any conclusions on the chemical mechanism of the tryptophan-nicotinamide conversion. The similarity of the action of abrin and that of tryptophan is paralleled by the findings of Gordon & Jackson (1935) that growing rats fed on a tryptophan-free diet can utilize abrin and suggests that in both cases a demethylation of the α-N atom takes place. The 2- and 5-methyltryptophans seemed to have an action antagonistic to tryptophan in Gordon & Jackson's experiments. The fact that, in the experiments with mixed cultures, nicotinamide synthesis was entirely inhibited in only two cases and partly in the four others might be due to the fact that, in the mixed culture, part of the methyltryptophans might have been used up by non-coliform bacteria.

In lower concentrations small differences in inhibition of nicotinamide synthesis by the various methyl derivatives were observed; but they were too small to justify the drawing of any conclusion on the relation between degree of inhibitory action and molecular structure, as discussed by Fildes & Rydon (1947) for *Bact. typhosum*. The inhibition of the nicotinamide formation by the 2-, 4-, 5- and 7-methyltryptophans, in the light of the theory of the action of metabolite antagonists as formulated by Fildes (1940), suggests that tryptophan is normally involved in this process. The mode of this action is obscure and needs further investigation. In a recent paper Heidelberger, Gullberg, Morgan & Lepkowsky (1948) have shown that after administration of DL-tryptophan, labelled with ^{14}C in the β position, to rabbits, dogs and rats the urine contained labelled kynurenine and kynurenic acid. The nicotinamide methochloride isolated from the urine of all three species was not radioactive. It was surprising that this compound was isolated from the urine of rabbits since these animals are known not to synthesize this compound in normal circumstances (for literature, see Ellinger & Abdel Kader, 1949*a*). These findings, combined with the findings of this paper, suggest the possibility that the nicotinamide-saving action of tryptophan is not due to a direct conversion, but to a stimulating effect of tryptophan on the biosynthesis mechanism. If this supposition should prove to be correct it would entirely change our conception of the nicotinamide-saving action of tryptophan, and attribute to tryptophan a catalytic coenzyme-like action on the enzyme system involved in the synthesis of nicotinamide.

The difference in the action of tryptophan on the nicotinamide synthesis by mixed cultures of intestinal bacteria on one hand and that by pure cultures of *Bact. coli* on the other (Ellinger & Abdel Kader, 1949*b*) could be explained in the following way: pure *Bact. coli* produces sufficient tryptophan to maintain the optimum rate of nicotinamide synthesis, while in mixed cultures non-coliform organisms consume so much tryptophan that extra tryptophan is needed for a maximum formation of nicotinamide. The inhibition of the growth of *Lb. arabinosus* by 7-methyltryptophan alone of all methyltryptophans examined is also obscure. The tryptophan-like action of abrin might be due to the greater instability of this compound which is probably demethylated easily to tryptophan.

SUMMARY

1. The effect of 2-, 4-, 5- and 7-methyltryptophans and of abrin on growth and nicotinamide formation by pure cultures of *Bacterium coli* (*Escherichia coli*)

and mixed cultures of rat caecum contents and on the acid production by *Lactobacillus arabinosus* has been studied.

2. Growth of *Bact. coli* or of mixed cultures was not markedly affected by any one of the methyltryptophans in the concentrations used; that of *Lb. arabinosus* was considerably inhibited by the 7-methyl compound.

3. Nicotinamide synthesis from ornithine or ammonium lactate by *Bact. coli* was completely inhibited by 2 mM and correspondingly less by lower concentrations of 2-, 4-, 5- and 7-methyltryptophans, but not by abrin. Nicotinamide synthesis by mixed bacteria from the rat caecum contents was either completely or partly inhibited by the former at 2 mM and not affected by the latter compound. These findings suggest that tryptophan is involved in the bacterial biosynthesis of nicotinamide.

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The Linkage of Glutamic Acid in Protein Molecules

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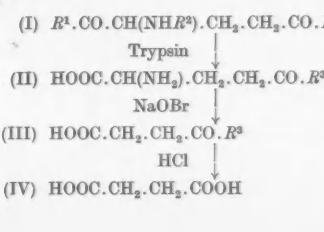
The experiments presented in this paper were carried out to answer the question as to whether the γ-carboxyl groups of glutamic acid are involved in the formation of bonds in protein molecules. The possible existence of such bonds in proteins is suggested by the fact that γ-linked glutamic acid residues have been found in glutathione, in the capsular substance of *Bacillus anthracis* (Bovarnick, 1942; Hanby & Rydon, 1946) and in folic acid (Boothe, Mowat, Hutchings, Angier, Waller, Stokstad, Semb, Gazzola & Subbarow, 1948). Different modes of combination of the γ-carboxyl groups of glutamyl residues in proteins can be considered: (1) formation of a peptide bond with the terminal amino group of a peptide chain, (2) ester linkages with hydroxyl groups of hydroxy amino-acids and (3) thio-ester linkages with cysteine molecules of a peptide side chain (Chibnall, 1942). Since the γ-substituted glutamyl residues could give rise to branching of the main peptide chain, the problem of γ-substitution is of great importance.

In order to test proteins for the presence of γ-substituted glutamyl residues we subjected them to the following series of procedures: (*a*) partial digestion with trypsin, (*b*) oxidation, (*c*) extraction of the acidified solution with ether (ether extract 1), (*d*) total hydrolysis, (*e*) extraction of the acid hydrolysate with ether (ether extract 2) and (*f*) determination of succinic acid in the last ether extract. Succinic acid found in ether extract 2 was considered as originating from the γ-glutamyl residues. The above mentioned method is based on the fact that the γ-peptide bond of glutathione is more resistant to trypsin than are the normal α-peptide bonds (Grassmann, Dyckerhoff & Eibeler, 1930; Kendall, Mason & McKenzie, 1930). It was expected, therefore, that at least a part of the α- and γ-substituted glutamyl residues (formula I) would be transformed into γ-glutamyl peptides (formula II) by the action of trypsin.



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The reactions (*b*)–(*e*), by which the γ-glutamyl peptide (II) is oxidized to the succinyl peptide (III) and hydrolyzed to succinic acid (IV), have been used by Quastel, Stewart & Tunnicliffe (1923) and by Kendall *et al.* (1930) in their work on glutathione. Their methods had to be modified because of the small amounts of succinic acid to be expected.

EXPERIMENTAL

Partial hydrolysis with trypsin. Casein (Merck) and edestin (Schuchardt) were commercial preparations. Ovalbumin was prepared according to Kekwick & Cannan (1936), serum albumin from ox serum by fractional precipitation with $(NH_4)_2SO_4$, and haemoglobin from horse blood by the ethanol method (Hoppe-Seyler, 1878). Since native ovalbumin and serum albumin are scarcely attacked by trypsin, these proteins were denatured by keeping their aqueous solutions in a boiling water bath for 30 min. We are grateful to Dr H. N. Rydon (Birkbeck College, University of London) for a sample of the capsular substance of *B. anthracis*. Each of the proteins (2 g.), dissolved or suspended in water, was mixed with 0·2 g. of trypsin (Merck) and 1 ml. of toluene. The pH was adjusted to 8·5 by the addition of N-NaOH and the volume brought to 100 ml. by adding water. The mixtures were kept at 38° for 24 hr., those containing the albumins for 48 hr. The pH was kept near 8·5 by adding small amounts of N-NaOH from time to time. The rate of hydrolysis was determined in control tests run under the same conditions with 200 mg. of protein and 20 mg. of trypsin. At 0, 24 and 48 hr., 2 ml. of neutralized 30% (w/v) formaldehyde and 0·2 ml. of a 1% solution of phenolphthalein were added and the amount of hydrolysis determined by titration with 0·1 N-NaOH. The volumes of NaOH recorded in Table 1 include those added to the protein samples to maintain the pH at 8·5.

Table 1. *Action of trypsin on proteins*

(Protein (200 mg.); trypsin (20 mg.); extent of hydrolysis determined by formol titration using 0·1 N-NaOH. Figures in brackets show increases in NaOH titres over those at zero time.)

Protein	0·1 N-NaOH used for neutralization		
	0 hr. (ml.)	24 hr. (ml.)	48 hr. (ml.)
Casein	2·8	8·5 (5·7)	10·8 (8·0)
Edestin	0·64	6·5 (5·9)	7·6 (7·0)
Ovalbumin	0·85	4·9 (4·0)	7·2 (6·3)
Serum albumin	2·2	6·5 (4·3)	8·0 (5·8)

The amount of the capsular substance of *B. anthracis* was too small to permit formol titration. All of this substance (50 mg.) was treated with 5 mg. of trypsin in 5 ml. of water for 24 hr.

Oxidation. The tryptic hydrolysate from 2 g. of protein was mixed at 0° with 240 ml. of a cold 1% (v/v) solution of Br_2 in 0·5 N-NaOH. After 5 min. the mixture was acidified with glacial acetic acid, and 20 g. of KI were immediately added. The volume of the solution was reduced to 40–50 ml. by evaporation *in vacuo* and HCl was added until congo red paper gave a blue colour. The solution was extracted continuously with ether for approximately 100 hr. (ether extract 1). It was then mixed with 2 vol. of concentrated HCl and refluxed for 4 hr. The hydrolysate was evaporated to a small volume, diluted with water and extracted with ether for 15 hr. (ether extract 2).

Determination of succinic acid. The ether extracts were evaporated, dissolved in a small volume of water, filtered and evaporated to dryness. To the residues, dissolved in 1 ml. of water, an excess of finely powdered $Ba(OH)_2.8H_2O$ and 9 ml. of ethanol were added. The insoluble Ba succinate was centrifuged, dissolved in dilute HCl, evaporated to dryness and extracted repeatedly with 3–5 ml. of ether. The ether extracts were evaporated, dried, weighed and analyzed for their succinic acid content according to Krebs (1937), washed pigeon-breast muscle being used as source of enzyme. The results of the analyses are shown in Table 3.

Control experiments. In Exps. 1–7 and 10 (Table 4) no trypsin was used. The substrates were oxidized directly by NaOBr. Casein (Exp. 1) was dissolved in 100 ml. of water by adding the required amount of N-NaOH. Casein hydrolysate (Exp. 2) was obtained by boiling 0·5 g. of casein with 20 ml. of 20% (w/v) HCl for 4 hr., concentrating and neutralizing the aqueous solution. In Exps. 8 and 9 the substrates were treated with one-tenth of their weight of trypsin and kept at 38° for 24 hr. The amino-acid mixture (Table 4, Exp. 10) contained 100 mg. of each of the following: glycine, alanine, serine, cystine, valine, leucine, isoleucine, aspartic acid, phenylalanine, tyrosine, arginine and histidine, together with 50 mg. of lysine, 50 mg. of tryptophan and 100 mg. of glucose. In all these experiments (see Table 4) the oxidation, extraction and hydrolysis with HCl were carried out as described in the preceding sections. In another control experiment glutamic acid (100 mg.) was boiled with 20 ml. of 23% (w/v) HCl containing 1 g. of KI and approximately 0·1 g. of I_2. After 4 hr. the solution was extracted continuously with ether and the extract examined in the usual manner. No succinic acid was found.

Determination of glutamine in the tryptic hydrolysate. Each of the hydrolysates (0·5 ml. equivalent to 10 mg. of protein) was brought to pH 3·5 by the addition of 0·1 N-HCl and kept

Table 2. *Determination of glutamine and of pyrrolidone carboxylic acid in the tryptic hydrolysate*

Protein	Consumption of 0·04 N-HCl		Found in 2 g. of protein		
	Before heating (ml.)	After heating (ml.)	Pyrrolidone carboxylic acid (mg.)	Glutamine (mg.)	Total glutamic acid (mg.)
Casein	0·025	0·060	26	41	440*
Edestin	0·020	0·062	21	49	410*
Ovalbumin	0·106	0·190	109	99	320*
Serum albumin	0·011	0·035	11	28	340†
Haemoglobin	0·010	0·032	10	26	170‡

* Chibnall (1946). † Shemin (1945). ‡ Foster (1945).

in a boiling water bath for 2·5 hr. The amount of NH_3 present before and after boiling was determined according to Conway (1933), 0·04 N-HCl being used for the titration of the NH_3 absorbed by the boric acid (Table 2). Asparagine is not split noticeably under these conditions (Chibnall & Westall, 1932).

RESULTS

Table 1 shows that 35–40 % of the peptide bonds of the proteins examined were split by the action of trypsin under the conditions of our experiments. Since γ-glutamyl bonds are slowly hydrolyzed by water at 38°, the duration of the digestion was reduced by employing relatively large amounts of trypsin. Table 4 (Exp. 9) shows that a considerable part of the glutathione resisted hydrolysis under the same conditions, but that another part was split as shown by the yield of succinic acid in ether extract 1.

The oxidizing agents used by Quastel *et al.* (1923) and by Kendall *et al.* (1930) were hydrogen peroxide, hypobromite or chloramine T. When the methods of these authors were applied to proteins, large amounts of succinic acid were found in the ether extract 2 (Haurowitz & Vardar, 1944). Later investigations showed that the destruction of hydrogen peroxide and of hypobromite is not complete in these methods, so that a further oxidation occurs during the hydrolysis with hydrochloric acid (Schwerin, Kara & Tanasoglu, unpublished results). Much lower results were obtained with chloramine T, which can easily be removed from the reaction mixture; but since the action of chloramine T requires temperatures of 40°, and since γ-glutamyl bonds are unstable at these temperatures in acid solutions, we preferred to use hypobromite at 0°. Goldschmidt & Strauss (1929) removed the excess of hypobromite by adding the calculated amount of hydrogen peroxide. It is hardly possible, however, to avoid a small excess of either peroxide or hypobromite. We therefore destroyed the peroxide by an excess of potassium iodide added in slightly acid solution. The bulk of the iodine formed was removed by the first ether extraction, so that it could not act as an oxidant during the subsequent acid hydrolysis.

Table 3 shows that 2 g. of protein on oxidation furnished 8–29 mg. of succinic acid before hydrolysis with hydrochloric acid (ether extract 1), and only 0·8–1·7 mg. of succinic acid after hydrolysis with acid (ether extract 2). The succinic acid found in ether extract 1 originates from the oxidation of glutamic acid and glutamine, both present in the tryptic hydrolysates of proteins. Only the succinic acid in ether extract 2 can arise from γ-glutamyl peptides of the formula II.

In view of the small quantities of succinic acid found in the ether extracts 2, it was very important to remove all of the succinic acid which had been

Table 3. *Determination of succinic acid in partially digested and oxidized proteins before and after hydrolysis with hydrochloric acid*

		Ether extract 1 (before HCl hydrolysis)			Ether extract 2 (after HCl hydrolysis)		
			Succinic acid in			Succinic acid in	
Protein	Wt. (g.)	Wt. (mg.)	2 g. of protein (mg.)	10^5 g. of protein (mol.)	Wt. (mg.)	2 g. of protein (mg.)	10^5 g. of protein (mol.)
Casein	2·0	88	29	12	15	1·5	0·64
Edestin	2·0	121	24	10	18	1·1	0·47
Ovalbumin	2·0	92	14	6	30	0·8	0·34
Serum albumin	2·0	138	23	10	12	1·1	0·47
Haemoglobin	2·0	120	8	3	39	1·7	1·4
Capsular substance of *B. anthracis*	0·05	3	0·3	5	1·6	0·48	8·1

Table 4. *Determination of succinic acid in control experiments*

Exp. no.	Substrate	Wt. (g.)	Hypobromite solution added (ml.)	Ether extract 1		Ether extract 2	
				Total wt. (mg.)	Succinic acid (mg.)	Total wt. (mg.)	Succinic acid (mg.)
1	Casein	5·0	200	5	0·4	6	0·6
2	Casein hydrolysate	0·5	190	85	37	0	0
3	Glutamic acid	0·09	24	63	45	0	0
4	Proline	0·05	5	3	1·0	0·4	0
5	Hydroxyproline	0·1	10	0·4	0	0	0
6	Methionine	0·1	27	—	0·12	0	0
7	Pyrrolidone carboxylic acid	0·2	20	9·7	4·6	0·6	0·07
8	Glutamine	0·1	26	28	18	0·97	0·01
9	Glutathione	0·128	30	9·4	4·7	11·2	8·0
10	Amino-acid mixture	1·4	140	35·7	2·0	—	—

formed before hydrolysis. For this reason the extraction with ether was continued for several days, until the extracts were free even of traces of succinic acid. Under these conditions no succinic acid passed into ether extract 2 after the oxidation and hydrolysis of glutamic acid, proline, hydroxyproline, methionine and of the amino-acid mixture examined (Table 4, Exps. 2–6). On the other hand, glutathione gave rise to large amounts of succinic acid in extract 2.

Glutamine and pyrrolidonecarboxylic acid gave traces of succinic acid in ether extract 2 (Table 4, Exps. 7 and 8). It was necessary, therefore, to determine both these substances in the tryptic hydrolysates. Since pyrrolidonecarboxylic acid is formed from glutamine, its amount is approximately equivalent to the quantity of ammonia found before heating at pH 3·5, while the amount of glutamine is equivalent to the ammonia formed during the heating. According to Table 2 the amount of pyrrolidonecarboxylic acid varies from 10 to 109 mg., that of glutamine from 26 to 99 mg./2 g. of protein digested. The amounts of succinic acid to be expected from these quantities of glutamine and of pyrrolidonecarboxylic acid, according to Table 4, are so small that they would not noticeably affect the values of succinic acid passing into the ether extract 2 (Table 3).

DISCUSSION

The amount of succinic acid found after hydrolysis of the oxidized peptides is very small (Table 3). If molecular weights of 100,000, 309,000, 44,000, 68,000 and 68,000 are assumed for casein, edestin, ovalbumin, serum albumin and haemoglobin respectively, the number of γ-glutamyl bonds per protein molecule would be 0·64, 1·45, 0·15, 0·32 and 0·95. These figures, however, have to be considered as minimum values. The actual number of γ-glutamyl bonds is certainly higher for the following two reasons: (*a*) a considerable part of the γ-glutamyl bonds is split during the treatment with trypsin at 37°, as shown by the experiment on glutathione (Table 4); (*b*) less than one molecule of succinic acid is formed, when glutamic acid or glutathione are oxidized (Table 4, Exps. 3 and 9). We are thus unable to calculate the real number of γ-glutamyl bonds per protein molecule, and we have to content ourselves with the result that such bonds are present and that their number in the proteins examined is very low. The assumptions of Chibnall (1942) and of Haurowitz & Vardar (1944), that some of the γ-carboxyl groups of glutamic acid are linked to other groups in protein molecules, are supported by these results. Much larger quantities of succinic acid were found in the ether extracts 2 of the capsular substance of *B. anthracis*. According to Hanby & Rydon (1946) this substance has a molecular weight of 50,000 or more, and is built up exclusively of chains of 50–100 α-glutamyl residues interlinked by γ-glutamyl bonds. Our results agree with this view. The problem of chain branching is not solved by our results for the γ-linked glutamyl residues can be bound according to the formula $R.NH.CH(COOH).CH_2.CH_2.CO.R$, without any ramification of the peptide chains.

SUMMARY

1. Casein, edestin, ovalbumin, serum albumin, haemoglobin and the capsular substance of *Bacillus anthracis* were partially digested with trypsin, and the resulting peptide mixture was tested for the presence of γ-glutamyl peptides by oxidizing it with sodium hypobromite. Succinic acid was removed by ether extraction and the succinyl peptides, insoluble in ether, were hydrolyzed by hydrochloric acid.

2. The amounts of succinic acid obtained by this procedure indicate the presence of a small number of γ-glutamyl bonds in the proteins examined, and of a much higher number of such bonds in the capsular substance of *B. anthracis*.

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